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CORRECTIONS.

On page 221, Vol. lxxiii, No. 1, May, 1927, line 9, read *5.812 gm.* for *5.812 mg.;* line 12, read *2.4 gm.* for *2.4 mg.*

STUDIES ON KETOSIS IN THE RAT.*

By HAROLD LEVINE AND ARTHUR H. SMITH.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)

Growth at the normal rate has been observed in rats on diets extremely rich in any one of the proximate principles. Osborne and Mendel (1920–21), Frank (1922), Drummond and Coward (1921), Smith and Carey (1923–24), and Jackson (1925) have been able to obtain normal growth in rats on diets, the energy of which was derived largely from either fat, protein, or carbohydrate. Animals likewise have been grown at the normal rate from 30 to 180 gm. in body weight on a ration practically devoid of preformed carbohydrate and containing 86 per cent of the total calories in the form of fat (Levine and Smith, 1927).

The ability of the rat to utilize such a fat-rich diet is in marked contrast to that of man. According to current views (Woodyatt (1921), Wilder and Winter (1922), Shaffer (1922), and Ladd and Palmer (1923)) a more or less definite ratio must exist between the fatty acid-forming or ketogenic substances and the glucose-forming or antiketogenic constituents in the diet if ketosis is to be averted in man. Shaffer has shown that a ketogenic-antiketogenic ratio of 2:1 in the diet represents the threshold beyond which abnormal amounts of acetone bodies appear in the urine.

With lower animals like the dog, rat, rabbit, goat, pig, and cow, however, such a definitely fixed ratio apparently does not apply, for it is well known that when diets are fed in which the 2:1 ratio is exceeded, or when these animals are subjected to fasting, acetone bodies fail to be excreted or appear in the urine only in small quantities. The literature reviewed below contains repeated evi-

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* The experimental data in this paper are taken from the dissertation submitted by Harold Levine in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1926.

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dence that would tend to throw some doubt on the wide applicability of the Shaffer theory of ketogenesis.

Geelmuyden (1897) working with dogs and rabbits was unable to bring about a ketosis either through the administration of a protein-fat diet or by means of fasting. He recorded 13 mg. of acetone per day as the highest output in the urine obtained in a dog on a high fat ration. In an interesting study of the susceptibility of various animals to acidosis, Baer (1904, 1906) showed that the rabbit, goat, and pig when fasted or fed a ration rich in fat do not excrete abnormal amounts of acetone. He also pointed out the difference in this respect between man and the ape.

Sjollema and Van der Zande (1923) in studying a peculiar disease in which cows suffered from acetonemia, were also unable to provoke a ketosis by dietary means. These workers concluded that fat and sugar metabolism are regulated from two independent centers—a view denying the existence of a reaction between some fragment of the glucose molecule and acetoacetic acid. They supported this contention by showing that the diseased cows had a normal carbohydrate metabolism, as evidenced from blood studies, whereas there was a decidedly abnormal fat metabolism.

In numerous experiments on normal dogs, Allen (1923 a, b, c) was unable to bring about a ketosis when high fat diets were fed or when fasting was resorted to. This investigator maintained that the diet is not the determining influence, nor is ketosis governed merely by two known factors, glucose $versus$ fatty acid, but that there is a third variable, namely the living organism. He pointed out further that "the dog seems to produce acetone less freely than man, not because different proportions of fatty acid and glucose are burned, but because the canine organism seems to deal with these mixtures differently than the human organism."

As a result of fasting experiments on two steers, Carpenter (1925) concluded that "these animals, like the dog and cat, exhibit no tendency toward ketosis during fasting. Apparently the keto antiketogenic ratio holds for man but not for these animals." He also suggested that there is another factor in ketosis other than a mere relation between fatty acid and glucose. A pig fed a high fat ration having a ketogenic ratio as high as 7:1 (calculated according to Shaffer's method) excreted no abnormal amounts of acetone bodies in the urine (Lueg and Flaschenträger, 1925). Since the blood sugar level of this animal when fed butter and butyric acid was 0.094 per cent as contrasted with a value of 0.072 per cent when the animal was fasted, they concluded that sugar was formed from fat. In metabolism studies on the rat Wigglesworth (1924) fed various diets including a highly ketogenic ration of butter fat and salts. It was found that in the rat, the normal urinary excretion of acetone plus acetoacetic acid (as acetone) ranged from 0.0 to 0.30 mg. per day and of β-hydroxybutyric acid from 0.8 to 2.5 mg. per day. He concluded that it is difficult to reconcile his results with those of the Shaffer (1921) theory of ketogenesis.

urine other than the acetone bodies was made. This procedure was necessary since substances in the urine such as lactic acid, uric acid, and creatine also give precipitates with the reagents used in determining the acetone bodies. Accordingly, the effect of these constituents was determined separately and then deducted from the total acetone bodies determination. The resultant value then gave the true acetone bodies excretion.

Besides making determinations for the total acetone bodies occasionally a differential analysis for acetone plus acetoacetic acid (as acetone) and β-hydroxybutyric acid (as acetone) was carried out. This procedure made it possible to ascertain what proportion of the total acetone bodies was excreted in the form of β-hydroxybutyric acid by the rat on the various dietary régimes.

Since the content of the urine ranged, on the average, from 4 to 12 mg. of total acetone bodies per 4 day period, single, instead of duplicate determinations were made because it was considered inadvisable to attempt to determine fractions of a mg. This procedure necessitated the employment of dilutions different from those in the original method of Van Slyke (1917) which was designed primarily for the determination of acetone bodies in human urine. All the analyses reported, with a few exceptions, are the result of single determinations. However, the method was checked frequently in duplicate determinations by analyzing a known mixture of the three acetone bodies, made up in such a manner that the proportions and amounts of the constituents were comparable to those present in the urine of the rat. Again, when it was demonstrated in the experiments in which alkali was administered and also in the phlorhizin experiments that the total acetone bodies excretion was unusually high, duplicate determinations were made and satisfactory checks obtained. Besides checking up the procedure in the ways mentioned above, a known amount of acetone bodies added to rat urine was recovered.

It was shown that contaminating foods, feces, or mineral oil had no effect on the analytical results. In the present study, the use of toluene or copper sulfate, as recommended by Van Slyke, proved objectionable so that recourse was had first to boric acid and later to sodium fluoride in 3 per cent solution. Neither of these preservatives had any effect on the determination.

In the earlier part of the work, the precipitate formed in the

TABLE I.

Composition of Diets.

Composition.	Calories per kilo of food.	Apportionment of total calories.
Fat Diet I.*		
per cent		*per cent*
Casein†.................. 25	·1025	Protein................... 13.8
Lard.................... 64⎫		
Cod liver oil............ 5⎬	6417	Fat....................... 86.2
Salts.‡.................. 6		
100	7442	100.0
Fat Diet II.*		
Casein.................. 23.5	964	Protein.................. 13.8
Starch.................. 11.9	488	Carbohydrate........... 6.3
Lard.................... 54.0⎫		
Cod liver oil............ 5.0⎬	5487	Fat....................... 79.9
Salts................... 5.6		
100.0	6939	100.0
Standard Diet III.*		
Casein................. 18	738	Protein.................. 13.8
Starch.................. 51	2091	Carbohydrate........... 39.2
Lard.................... 22⎫		
Cod liver oil........... 5⎬	2511	Fat....................... 47.0
Salts.................. 4		
100	5340	100.0
Carbohydrate Diet IV.§		
Casein................. 14.0	514	Protein.................. 13.8
Starch................. 79.0	3239	Carbohydrate........... 77.8
Cod liver oil........... 3.8	353	Fat....................... 8.4
Salts................... 3.2		
100.0	4168	100.0
Fat-NaHCO₃ Diet V.§		
Casein................. 23.5	964	Protein.................. 14.9
NaHCO₃............... 11.9		
Lard.................... 54.0⎫		
Cod liver oil........... 5.0⎬	5487	Fat....................... 85.1
Salts................... 5.6		
100.0	6451	100.0

TABLE I—*Concluded.*

Composition.	Calories per kilo of food.	Apportionment of total calories.
Protein Diet VI.§		
per cent		*per cent*
Casein.................. 96.0	3936	Protein................. 100.0
Salts................... 4.0		
5 drops cod liver oil added daily apart from food.		
100.0	3936	100.0

* 40 mg. yeast vitamin powder (Harris) fed daily apart from the food. The potency of this particular sample had been proved in this laboratory.

† A fat-free product, No. 453, from the Casein Manufacturing Company, 13 Park Row, New York. It was washed with water of pH 4.6, then with alcohol (95 per cent) and dried in a steam drier. By this process, the protein content (N × 6.25) was raised to approximately 90 per cent (air-dry basis) while the ash content was reduced to about 1 per cent.

‡ The inorganic salt mixture described by Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxii, 374).

§ 125 mg. dried yeast fed apart from the food. Obtained from the Northwestern Yeast Company, Chicago, and tested for vitamin B potency in this laboratory.

determination of the acetone bodies was weighed. Later, titration of the mercury content of the precipitate was carried out. It was found that the latter procedure proved just as accurate as the former.

In a few of the experiments, total nitrogen was determined by the Kjeldahl method.

Composition and Preparation of Diets.—In Table I is shown the nature of the six different diets employed in the investigation. The high carbohydrate diet (No. IV) was made up by stirring the dry ingredients and the cod liver oil into the required amount of boiling water. This treatment did not reduce the vitamin A potency of the food below the requisite concentration for optimal growth. The moist material was then spread in thin layers in pans and dried to a crisp cracker in a steam drier, the starch being partially dextrinized by this treatment; the dried food was then ground to a fine powder.

The high protein diet (No. VI) was made up by mixing the casein and the salts together. In addition, the animals on this ration received 5 drops of cod liver oil daily apart from the food.

Most of the diets (Nos. I to IV) were so planned that regardless of the calorie value of the food, the ratio of protein calories to the total calories would be the same as that of Standard Diet III. Furthermore, in most rations (Diets I to IV) the ratio of salt content to the total calories was constant. The adjustment of intake to the calorie value of the food could therefore be made by the rats without disturbing the actual consumption of the two indispensable factors; namely, the inorganic salts and the protein.

All the fat diets (Nos. I, II, and V) and Standard Diet III were prepared as follows: The dry ingredients were first mixed and then added to the melted lard in a large evaporating dish. The cod liver oil was immediately added and the whole thoroughly mixed. After admixture, the food was placed in the ice box to allow the lard to solidify, care being taken further with the high fat rations to stir the ingredients thoroughly *just* before the setting of the lard.

Output of Acetone Bodies on Various Diets.

The excretion of acetone bodies on various diets rich in either protein, fat, or carbohydrate was compared with that on a well balanced ration so that the effect of a highly ketogenic or antiketogenic diet could be ascertained. Calculation of the *expected* excretion of acetone bodies by means of the factors used by Shaffer (1922) made it possible to make a comparison with the *actual* output of some of the rats. The effect of suddenly changing from an antiketogenic ration to a ketogenic diet was also studied.

Experimental Results.—In a few experiments, rats that had exhibited normal growth on Fat Diet I for a considerable length of time were immediately transferred to metabolism cages. Thus, the amounts of acetone eliminated by these rats could be compared with the output of animals that were *suddenly* transferred from a stock diet to Fat Diet I. Most of the animals, however, were allowed to become adjusted to both the diet and the cage for several days before the urine collections were begun. The diets used in the present experiments were as follows: Standard Diet

III, Fat Diet I, Carbohydrate Diet IV, and Protein Diet VI (see Table I).

Records of the body weight, the food intake, in some cases the nitrogen in the food and the output in the urine, and values expressing the mg. of total acetone bodies (as acetone) excreted in successive experimental periods of 4 days each were kept.

A condensed summary (Table II) derived from the data in the protocols mentioned above is given below. From a study of this table, it is apparent that not only is there considerable variation in each of the four groups of rats on the four different diets but also in individual animals in successive periods. Wigglesworth (1924) in his studies on ketosis in the rat reported similar variations.

As seen in Table II most of the values ranged from 4 to 12 mg. for a 4 day period; 1 to 3 mg. as a daily output. In this connection, the data obtained by Wigglesworth (1924) and by Cori and Cori (1927) on the rat are of interest in comparing the above results. Wigglesworth reports a daily excretion of 0.0 to 0.3 mg. of acetone plus acetoacetic acid (as acetone) and 0.8 to 2.5 mg. of β-hydroxybutyric acid. In terms of acetone, this would give a daily output of 0.4 to 1.7 mg., a value slightly lower than that obtained in the present investigation. Wigglesworth, however, made no effort to prevent loss of acetone through volatilization, a fact which might explain the difference obtained between his results and those shown above. Again, he collected the urine every 24 hours in contrast to the 4 day period used in the present experiments. As Hopkins has pointed out, such a short period is undesirable because a proper demarcation of consecutive periods is impossible. Cori and Cori (1927) report an excretion of 0.4 mg. of acetone bodies per 100 gm. of body weight per 24 hours, equivalent to a daily output of 1 mg. for a rat weighing 250 gm.

From the values given in Table II, it is obvious that the excretion of total acetone bodies is independent of the proportion of fat, carbohydrate, or protein in the diet. In other words, ketosis in the rat apparently is not related to the organic portion of the diet for in the present experiments the acetone bodies excreted remained essentially the same whether the diet supplied the greater part of the energy as fat, carbohydrate, or protein. From the above experimental evidence, it appears that in the rat either the mechanism regulating the production of acetone bodies differs

TABLE II.

*Urinary Excretion of Acetone Bodies on Various Diets.**

Expressed as mg. of acetone per 4 day period.

Period No.

Rat No.

Standard Diet III.

56	9.9	3.7				
57	4.1	13.4				
58	7.2	8.1	9.6	10.3	8.4	
65	13.8	15.4	20.1	19.7	10.3	4.5
66	12.6	9.8	10.2	10.3		
67	39.6	15.0	10.6	9.2		
68	12.6	16.6		28.2		
69	19.1	6.3	12.0	11.8	12.7	

Average of above 31 determinations on 8 rats = 12.7 mg. total acetone bodies.

Protein Diet VI.

383	3.9	3.4	9.6	0.9
384	3.5	4.4		11.6
388	1.5	1.5	5.2	0.0
543	17.8	11.3	10.6	9.9
544	12.6	9.5	3.7	9.6
545	16.4	9.2	3.9	5.8
546	14.2	9.5	4.6	4.6
547	6.8	5.6	10.6	9.9

Average of above 31 determinations on 8 rats = 7.5 mg. total acetone bodies.

Carbohydrate Diet IV.

385	3.8	3.5	7.8	7.5
386	0.7	0.0	6.6	7.6
387	2.8	2.8	7.9	6.1
525	17.0	8.6		
526	18.5	12.5	12.5	35.3
527	17.4	11.6	31.5	24.6
528	9.2	9.1	4.6	5.8
529	6.5	6.7	5.3	5.0
530	10.3	6.7	6.1	12.3

Average of above 34 determinations on 9 rats = 9.8 mg. total acetone bodies.

TABLE II—*Concluded.*

Rat No.	Period No.						
	1	2	3	4	5	6	7
Fat Diet I.							
59	6.6	11.0	11.0	9.9	8.6		
60	7.9	9.6	9.0	12.7	21.5	14.0	11.8
61	10.1	11.6	11.8	9.7	11.1	9.7	10.2
62	6.6	7.2	8.6	7.1	7.7	13.7	10.8
63	10.0	10.8	13.3	12.6	18.0	6.7	9.5
64	9.7	9.0	10.8	14.2	13.0	13.8	15.1
363	4.6	5.0	3.2				
365	4.0	2.5	4.1				
366	4.8	3.0	4.2				
383	10.3	8.2	6.6				
385	11.9	5.6	8.5				
386	7.9	10.8	7.2				
387	5.8	4.1					
388	4.7	4.3	6.1				
418	27.6	9.9	4.1	7.1	5.5		
419	8.9	12.2	6.9	6.2	6.7		
420	5.9	10.8	5.6	5.9	3.6		
421	2.9	5.5	3.0	5.5	6.1		
422	6.6	6.8	4.8	5.0	6.4		
423	9.6	16.7	7.1	3.0			
526	11.2	13.6	18.5				
527	4.1	6.9	5.6				
528	2.7	4.7	4.9				
529	1.4	3.0	2.3				
530	3.5	6.3	5.4				
543	5.6	7.5	4.4				
544	5.6	5.6	5.6				
545	42.4	16.3	7.0				
546	2.5	5.6	4.6				
547	6.3	2.9	3.5				

Average of above 122 determinations on 30 rats = 8.1 mg. total acetone bodies.

Average of 218 determinations on 55 rats on the above four diets = 8.9 mg. total acetone bodies.

* For the composition of these diets see Table I.

from that in man or the principles underlying the theory of ketogenesis as generally accepted are untenable. The results obtained bear out those of Lueg and Flaschenträger (1925) and others who have worked on lower animals.

The *actual* excretion of acetone bodies of rats on Fat Diet I was then compared with the amounts *predicted* by the Shaffer method of calculation. According to the formula of Shaffer and of Ladd and Palmer, this diet is ketogenic and, if applicable to the rat, should give rise to the excretion of abnormal amounts of acetone bodies. The calculations illustrate the wide discrepancy between the *actual* and the *predicted* output.

If the factors given by Shaffer (1922) are employed together with a knowledge of the total metabolism, the amount of fat burned, and the urinary nitrogen, it is possible to calculate the ketogenic ratio and to determine the *expected* output of acetone bodies as β-hydroxybutyric acid in rats consuming Fat Diet I. Since this ration is practically devoid of preformed carbohydrate, the calculation is obviously simplified. The total metabolism in the present calculation is assumed to be 95 per cent of the calorie intake.[3] With this value, the amount of fat undergoing oxidation in the body can be determined fairly closely.

The accompanying calculation on Rat 60 serves to illustrate the method. This procedure allows the determination of the ketogenic ratio and also makes possible the evaluation of the *expected* excretion of acetone bodies as β-hydroxybutyric acid.

Rat 60.

Total metabolism.............................. = 106.0 calories.
Protein metabolized (urine N (0.502 gm.) ×
    26.5 calories)............................. = 13.3   "
Fat burned.................................... = 92.7   "

$$\frac{92.7}{9.3} = 10 \text{ gm. of fat burned.}$$

	Ketogenic millimols.	Glucose millimols.
Protein (0.502 gm. N)........................	7.53	10.04
Fat (10 gm.).................................	34.30	5.70
	41.83	15.74

[3] Rats on this high fat ration can utilize 98 to 99 per cent of the fat ingested (Levine and Smith, 1927).

$$\text{Ketogenic ratio} \quad = \frac{41.83}{15.74} = 2.7.$$

$$\text{``} \quad \text{balance} = 41.83 - 2\ (15.74).$$

$$= 10.35 \text{ excess ketogenic mols.}$$

$$10.35 \times 0.104 \quad = 1.08 \text{ gm. or } 1080 \text{ mg. of } \beta\text{-hydroxybutyric}$$
$$\text{acid } expected \text{ excretion. } \textbf{Actual} \text{ excre-}$$
$$\text{tion } 20.2 \text{ mg.}$$

The results of similar calculations applied to Rats 61 to 64 are shown in Table III. This table is striking in that it shows a wide difference between the actual and the expected excretion of acetone bodies.

The output of acetone bodies in relation to changes in the diet was next studied. When the human subject consumes a diet

TABLE III.

Rat No.	Actual caloric intake.	Total metabolism.	Ketogenic value of mixture metabolized.	Urine nitrogen.	Total acetone bodies excretion as β-hydroxybutyric acid.	
					Found.	Expected.
	calories	*calories*		*gm.*	*mg.*	*mg.*
60	111	106	2.7	0.502	20.2	1080
61	141	134	2.7	0.600	18.4	1539
62	148	141	2.8	0.610	19.4	1596
63	141	134	2.5	0.696	17.0	1144
64	141	134	3.0	0.667	27.2	2600

which contains a large amount of either carbohydrate or protein, unusual amounts of acetone bodies are not excreted in the urine. According to the Shaffer theory of ketogenesis, such diets are anti-ketogenic in nature and hence should not give rise to abnormal amounts of acetone bodies. When the diet is changed suddenly from an antiketogenic ration to one highly ketogenic, such as a fat diet, for a short period of time, no change in the acetone bodies excretion occurs, owing to the fact that the glycogen store in the body has not yet been exhausted and is therefore able to exert its antiketogenic effect. Subsequently, however, when the glycogen supply is depleted, the ketogenic action of the fat prevails and acetone bodies make their appearance in the urine in unusual amounts. The foregoing facts have been confirmed by several investigators working on man.

On the lower animals, little work has been done in this particular field of experimentation, probably because in the lower mam-

TABLE IV.

Output of Acetone Bodies in Relation to Changes in Diet.

Expressed as mg. of acetone per 4 day period.

Rat No.	Period No.						
	1	2	3	4	5	6	7

Change from Protein Diet VI to Fat Diet I.

	Protein diet.				Fat diet.		
383	3.9	3.4	9.6	0.9	10.3	8.2	6.6
388	1.5	1.5	5.2	0.0	4.7	4.3	6.1
543	17.8	11.3	10.6	9.9	5.6	7.5	4.4
544	12.6	9.5	3.7	9.6	5.6	5.6	5.6
545	16.4	9.2	3.9	5.8	42.4	16.3	7.0
546	14.2	9.5	4.6	4.6	2.5	5.6	4.6
547	6.8	5.6	10.6	9.9	6.3	2.9	3.5

Change from Carbohydrate Diet IV to Fat Diet I.

	Carbohydrate diet.				Fat diet.		
385	3.8	3.5	7.8	7.5	11.9	5.6	8.5
386	0.7	0.0	6.6	7.6	7.9	10.8	7.2
387	2.8	2.8	7.9	6.1	5.8	4.1	
526	18.5	12.5	12.5	35.3	11.2	13.6	18.5
527	17.4	11.6	31.8	24.6	4.1	6.9	5.6
528	9.2	9.1	4.6	5.8	2.7	4.7	4.9
529	6.5	6.7	5.3	5.0	1.4	3.0	2.3
530	10.3	6.7	6.1	12.3	3.5	6.3	5.4

Change from Standard Diet III to Fat Diet I.

	Standard diet.				Fat diet.		
65	13.8	15.4	20.1	19.7	22.3		
66	12.6	9.8	10.2	10.3	15.5		
67	39.6	15.0	10.6	9.2	9.4		
68	12.6	16.6		28.2	15.7		
69	19.1	6.3	12.0	11.8	14.4		

mals there seems to be no relation between diet and ketosis. Working with dogs, Allen (1923, *b*) found that a preliminary period of feeding with a carbohydrate diet did not create a susceptibility

to ketosis when a fat diet was subsequently fed.   Lueg and Flaschenträger (1925) fed a carbohydrate diet, consisting of starch and sugar, to a pig and then suddenly changed to a ration of fat or fatty acids, with the result that only small amounts of acetone bodies appeared in the urine.

In the present experiments, the effect on the acetone bodies excretion of changing from an antiketogenic ration such as Protein Diet VI, Carbohydrate Diet IV, or Standard Diet III to a ketogenic diet such as Fat Diet I was determined.   In this way, it was possible that a preliminary feeding with a non-ketogenic ration would bring about a susceptibility to ketosis when subsequently a ketogenic diet was fed.

Table IV shows the result of the sudden dietary changes suggested above.   It is evident that the output of acetone bodies is not affected by sudden, marked changes in the diet.   Indeed, in many cases, the values on the fat ration are lower than those on the preliminary rations.

Output of Acetone Bodies in Fasting.

In contrast to man, the lower animals such as the dog, rabbit, goat, and cow seem to react differently, in that abnormal amounts of acetone bodies to not appear in the urine even after long periods of fasting.   Neither Waldvogel (1899), who fasted a dog for a period of 33 days, nor Carpenter (1925) who subjected two steers to fasting periods of 5, 7, 10, and 14 days were able to bring about a ketosis, observations repeatedly corroborated for various lower animals by others (Baer, 1904, 1906; Geelmuyden, 1897; Allen, 1923 *a*, *b*; Sjollema and Van der Zande, 1923).   Apparently, the lower animals are extremely resistant to ketosis even when a drastic strain such as fasting is put upon the organism.

Experimental Results.—Fattened rats weighing from 250 to 300 gm. were subjected to fasting for periods ranging from 4 to 7 days, in the course of which they lost approximately one-fourth of the body weight.   In some cases, Fat Diet I was fed for several days previous to the fasting period.

Table V gives the complete data pertaining to the nine rats that were subjected to fasting for varying lengths of time.   The values for the acetone bodies excretion are given both for the entire fasting period and for a calculated period of 4 days.

.The latter calculation was made so that a comparison of the acetone bodies excretion in fasting could be made with the out-. put of rats that were fed the various unusual rations previously mentioned. The results show that the values calculated on a 4 day basis do not differ significantly from those obtained when rats were fed the various unusual rations or a well balanced diet. Moreover, the nature of the diet fed previous to the fasting period does not seem to effect the subsequent excretion of acetone bodies.

In. connection with the results obtained here, those of Cori and Cori (1927) are of interest. They found that rats fasting in the summer excreted an average of 6.2 mg. of acetone bodies per 100

TABLE V.

Urinary Output of Acetone Bodies in Fasting.

Rat No.	Initial body weight.	Loss in body weight.	Period of fasting.	Total acetone bodies excreted during period of fasting.*	Total acetone bodies excretion calculated for 4 days.*
	gm.	*gm.*	*days*	*mg.*	*mg.*
65†	257	63	6	9.5	6.3
69†	266	55	6	13.4	8.9
74†	155	34	4	5.4	5.4
75†	145	22	4	6.1	6.1
100‡	278	45	7	33.7	19.2
101‡	265	39	7	24.2	13.8
102‡	250	41	7	29.5	16.9
103‡	260	44	7	10.4	5.9
104‡	195	44	7	4.1	2.3

* Expressed as acetone.

† Previous to fasting period this animal was fed dog biscuit diet.

‡ Previous to fasting period this animal was fed Fat Diet I.

gm. of body weight per 24 hours, whereas animals fasted in the winter months excreted an average of 1.9 mg. of acetone bodies per 100 gm. of body weight per 24 hours. When these data are calculated to the excretion per 4 days for a rat of 250 gm. body weight it is evident that such a seasonal variation did not occur in the present investigation. Rats 100 to 104 were subjected to fasting in the summer, while Rats 65, 69, 74, and 75 were fasted in the winter months.

The data here obtained seem to indicate that fasting in the rat does not produce an appreciable ketosis.

Influence of Alkali on Urinary Excretion of Acetone Bodies. .

It is well known that when alkali is fed to diabetic patients an increased excretion of acetone bodies results. The experiments of Geelmuyden (1901), Davies, Haldane, and Kennaway (1920–21), Hubbard (1922–23), Hubbard and Wright (1924–25), Booher and Killian (1923–24), Beumer and Soecknick (1924), and Haldane and Wigglesworth (1924) show that likewise in the normal human subject, abnormal amounts of acetone bodies appear in the urine when alkali is given.

Wigglesworth (1924) was able to bring about a distinct ketosis in rats both by injecting and by feeding sodium bicarbonate.

TABLE VI.

Influence of Alkali on Urinary Excretion of Acetone Bodies.

Expressed as mg. of acetone per 4 day period.

Rat No.	Period 1, Fat Diet II.	Period 2, Fat-Bicarbonate Diet V.	Period 3, Fat-Bicarbonate Diet V.	Period 4, Fat-Bicarbonate Diet V.	Period 5, Fat Diet II.	Period 6, Fat Diet II.
1	8.3	20.3	12.1	14.3	4.1	2.0
2	5.8	13.5	19.2	31.2	4.3	3.0
3	6.5	26.2	31.0	47.8	3.7	1.0
4	6.2	70.9	28.6	64.8	4.1	3.7
5	6.4	63.9	95.4	75.3	3.2	2.7
105	7.3	22.1	50.1*			
106	4.8	15.8	27.0*			
107	6.0	14.9	14.5*			
108	3.1	5.6	8.1*			
109	6.3	60.4	111.3*			

* This value represents the acetone bodies excretion in 5 days.

Calcium lactate, sodium acetate, and alkali-forming foods such as carrots also increased the output of acetone bodies in the urine. He also showed that acids had the reverse effect and could diminish considerably a ketosis due to alkali. Wigglesworth believes that alkali causes a real disturbance in the metabolism of fat, while other workers are of the opinion that alkali interferes with the normal oxidation of sugar. Either view suffices to explain the increased excretion of ketone bodies.

Experimental Results.—Fat-Bicarbonate Diet V (see Table I) was employed. This ration was practically devoid of preformed

carbohydrate and contained, in addition to a large proportion of fat, 11.9 per cent of sodium bicarbonate. This diet resembled Fat Diet II in all respects save that sodium bicarbonate was present in place of an equal amount of starch. A condensed table (Table VI) derived from data in the protocols is given. In this table are given values expressing the output of acetone bodies in mg. in a period of 4 days.

Previous to the alkali periods, all the rats were fed Fat Diet I to ascertain the acetone bodies output on this ration. In some cases, after the animals had consumed the alkali-containing ration for several periods, Fat Diet II was fed again to ascertain whether the acetone bodies excretion would subside and return to the level attained in Period 1.

It is obvious from the values given in Table VI that, in most cases, the presence of alkali in the diet had the effect of increasing the output of acetone bodies in the urine. When Fat Diet II was fed again in Periods 5 and 6, the acetone bodies excretion dropped to its former level.[4] ·

From the above results, it can be concluded that alkali (sodium bicarbonate) in a diet almost entirely lacking preformed carbohydrate causes a rise in the output of acetone bodies. Replacement of the alkali-containing ration with a similar ration containing starch in place of the alkali is accompanied by a drop in the acetone bodies excretion to its normal level. The results obtained confirm those of Wigglesworth (1924) in similar studies on the rat.

Influence of Phlorhizin on Excretion of Acetone Bodies.

Phlorhizin has been shown to increase the output of β-hydroxybutyric acid under certain conditions in dogs, rabbits, and goats (von Mering, 1889; Geelmuyden, 1898; Baer, 1904, 1906; Wolf and Osterberg, 1911; Sassa, 1914; Allen and Wishart, 1923). Few investigators, however, have attempted to reproduce the symptoms of phlorhizin diabetes in the rat probably because of the difficulties involved in making metabolic studies on this animal.

Asher and Calvo-Criado (1925) seem to be the only workers

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[4] Unpublished observations in this laboratory indicate that alkali added to a diet containing 75 per cent of the calories from carbohydrate likewise brings about a marked increase in the acetone bodies excretion.

to have made a study of the acetone excretion of the rat, under the influence of phlorhizin. Rats were depleted of their glycogen stores in the body by the administration of peptone and thyroid. After 5 to 6 days of this preliminary treatment, 50 mg. of phlorhizin in 1 cc. of olive oil were injected subcutaneously into each rat on alternate days. These investigators, in making a study of the conversion of fat to sugar, carried out determinations for sugar, nitrogen, and occasionally acetone and acetoacetic acid in the urine.

TABLE VII.
Influence of Phlorhizin* Injection on Excretion of Acetone Bodies.

Rat No.	Phlorhizin injected daily.†	Total acetone bodies excreted in 4 days.‡
Phlorhizin and Fat Diet II.		
	mg.	*mg.*
6	25	607.5
7	25	599.4
8	25	81.0
9	25	526.5
10	25	101.3
Phlorhizin and fasting.		
900	50	270.0§
901	25	290.0

   * Purified according to the method described by Deuel, H. J., Jr., and Chambers, W. H., *J. Biol. Chem.*, 1925, lxv, 7.

   † Phlorhizin (in 1 cc. of olive oil) injected subcutaneously.

   ‡ All urine samples gave a strong qualitative (Benedict's) test for sugar.

   § This value is for a 2 day period. This animal died on the 2nd day of this period apparently from an overdose of phlorhizin.

They found that the acetone output varied with different animals from 1 to 56 mg. daily. Apparently, however, no determinations for β-hydroxybutyric acid or total acetone bodies were made.

Experimental Results.—Rats were injected with phlorhizin during a period of fasting or while being fed Fat Diet II. Table VII gives the results of these experiments. Since it was necessary to determine, first of all, the maximum daily dosage of phlorhizin which, when injected over a period of at least 4 days, would not cause death, Rat 900 was given subcutaneously a daily dosage of

50 mg. of phlorhizin in 1 cc. of olive oil, with the result that death occurred in 2 days. Rat 901 was therefore injected with 25 mg. daily and at the end of 4 days was still alive. Accordingly, this dosage was used in the remaining experiments.

TABLE VIII.

Proportions of Acetone Bodies Excreted by the Rat under Various Dietary Régimes.

Rat No.	Determination* performed.			Total acetone bodies present as β-hydroxybutyric acid.
	Total acetone bodies. (1)	Acetone plus acetoacetic acid. (2)	β-hydroxybutyric acid. (3)	(4)
Fat Diet I.				
	mg.	*mg.*	*mg.*	*per cent†*
418	9.9	3.1	7.0	69
418	4.1	1.4	2.9	68
545	7.0	2.7	4.9	65
67	11.8	3.6	8.1	70
Protein Diet VI.				
388	3.4	1.2	2.2	65
543	10.6	3.6	7.0	67
Fasted.				
100	33.7	8.8	24.5	74
103	10.4	3.9	7.5	66
Fat Diet II and phlorhizin.				
10	101.3	29.3	68.8	70

* Results of all determinations are expressed as acetone.
† This value obtained from Columns 2 and 3 as follows:

$$\frac{(3)}{(2) + (3)} = \text{per cent of total acetone bodies present in the form of } \beta\text{-hydroxybutyric acid.}$$

It is evident from Table VII that the injection of phlorhizin into rats fed Fat Diet II or subjected to fasting results in the excretion of large amounts of acetone bodies and of sugar in the urine. The largest amount of acetone bodies was excreted by Rat 6 which eliminated in the urine 607.5 mg. in a period of 4 days. When the amounts excreted under the influence of phlorhizin are compared

with the amounts excreted by normal rats on various diets or during fasting, it is apparent that considerably higher values were obtained when phlorhizin was used.

Proportions of Acetone Bodies Excreted by the Rat under Various Dietary Régimes.

Various investigators (Kennaway, 1914; Moore, 1916; Labbé, Labbé, and Nepveux, 1921; Guillaumin, 1923; and Lublin, 1924) have made a study of the relative proportions of the acetone bodies excreted by various diabetic and non-diabetic patients under different dietary régimes. All of these workers agree that in man no fixed ratio exists between the amount of β-hydroxybutyric acid and of acetone plus acetoacetic acid eliminated in the urine.

Experimental Results.—In the present investigation it was ascertained, firstly, which one of the acetone bodies is excreted in the largest amount and, secondly, whether a constant proportion of β-hydroxybutyric acid is excreted by the rat. Table VIII shows the results of several differential analyses carried out simultaneously with many of the experiments previously described. The data show that β-hydroxybutyric acid formed the largest part of the acetone bodies excretion. The last column in Table VIII shows that rats when fed either Fat Diet II in conjunction with the injection of phlorhizin, Fat Diet I, or Protein Diet VI, or when subjected to fasting excreted a rather constant proportion (approximately 70 per cent) of β-hydroxybutyric acid. Cori and Cori (1927) reported similar values for fasting rats. It is apparent, therefore, that all of the dietary régimes mentioned above reacted alike in so far as the proportion of β-hydroxybutyric acid was affected.

SUMMARY AND CONCLUSIONS.

When rats were given various unusual diets containing a large amount of either fat, carbohydrate, or protein, or a well balanced ration, no essential difference in the amount to the acetone bodies excreted in the urine was observed, nor did an appreciable degree of ketosis appear. This result was surprising since the high fat ration used would, if metabolized in the human, give rise to an abnormal excretion of acetone bodies. In the rat, the excretion of

acetone bodies appears to be independent of the proportions of fat, carbohydrate, and protein in the diet.

Variations in the acetone bodies output were observed in different rats and in individual rats in successive experimental periods. On diets containing an extreme concentration of fat, carbohydrate, or protein, or a well balanced mixture of these constituents most of the values for a 4 day period ranged from 4 to 12 mg. and hence 1 to 3 mg. as a daily output.

From the experimental results cited above, it appears that in the rat either the mechanism regulating the production of acetone bodies is different from that in man or the rat can metabolize fats without the intermediation of carbohydrate. When the method of calculation as employed by Shaffer was applied to the metabolism of several rats on a ketogenic ration, it was found that a wide discrepancy existed between the actual and the predicted excretion of acetone bodies. The average output of acetone bodies excreted by five rats in a 4 day period was 20 mg., whereas calculated according to the theory of ketogenesis, an average output of 1592 mg. was expected.

Attempts to bring about a ketosis by resorting to a preliminary period of feeding with a high carbohydrate, high protein, or a standard diet and then suddenly changing to a high fat ration were unsuccessful. This sudden change in diet did not effect the level of the acetone bodies excretion.

Fasting for periods ranging from 4 to 7 days produced no appreciable ketosis in the rat in contrast to the human species. The output of acetone bodies in fasting was similar to that of animals consuming the various diets.

When sodium carbonate was added to a high fat diet, usually an immediate rise in the output of acetone bodies was observed. On changing back to the high fat diet without alkali, the acetone bodies excretion promptly fell to the former level.

The results with phlorhizin indicate that the nature of fat-carbohydrate oxidation mechanism of man and of the rat is similar qualitatively but that the quantitative aspect in the two species is different. The daily injection of phlorhizin into rats resulted in a distinct ketosis as well as in the excretion of sugar in the urine.

A rather constant proportion—about 70 per cent—of the acetone bodies was excreted in the form of β-hydroxybutyric acid by rats on many of the dietary régimes mentioned above.

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DIRECT PRECIPITATION OF CALCIUM IN HUMAN MILK.

By CARMEN S. ROTHWELL.

(From the Department of Pediatrics, Yale University, New Haven.)

In a previous communication (1) we showed that calcium could be directly precipitated from cow's milk with ammonium oxalate. Efforts to apply the same method to human milk, there reported, resulted in incomplete recovery of the calcium. In the present paper we desire to report that, by a slight modification of our original procedure, calcium may be quantitatively precipitated from human milk.

It is well known that the protein composition of the two milks differs. The protein of cow's milk is 80 per cent casein and 20 per cent albumin, whereas the protein of human milk consists of 40 per cent casein and 60 per cent albumin. Bechhold (2) suggests that this variation has much to do with their different reactions toward coagulation by acids or rennet, for the albuminates form irreversible colloids that protect against the reaction upon the caseinates. Porcher (3) and Grollman (4) have shown that the reaction is further complicated by the relative and absolute amount of the phosphates. It seems probable that a similar mechanism alters the precipitability of calcium in the two milks. Van Slyke and Bosworth (5) state that calcium caseinate is soluble in 5 per cent sodium chloride, and that sodium can replace calcium in basic caseinates. With this observation as a starting point, we have developed the following satisfactory procedure.

Shake the sample of human milk, and pipette 1 cc. into a 15 cc. tapered centrifuge tube. Add 2 cc. of 10 per cent sodium chloride and 1 cc. of saturated ammonium oxalate. This gives a final concentration of 5 per cent sodium chloride. Mix and let stand ½ to 1 hour. Centrifuge, pour off the supernatant milk and cream, and wash once with about ½ cc. of ether and 2 cc. of a solution containing 2 cc. of concentrated ammonia to 100 cc. of water.

Repeat the washing with 2 cc. of the ammonia solution alone, according to the technique of Clark and Collip (6). Add and pour off the wash fluid carefully, to avoid disturbing the precipitate, otherwise results may be low (7). Titrate with 0.01 N potassium permanganate in the usual manner.

For the analysis of colostrum, it is necessary to centrifuge 5 minutes at 1500 R.P.M. before pipetting the sample, in order to remove the mucus and cellular detritus; otherwise this material contaminates the precipitate and gives high results. Loosen the plug of cream from the sides of the tube and remove, together

TABLE I.

Effect of Varying Final Concentrations of NaCl on the Direct Precipitation of Calcium in Human Milk.

Values expressed as mg. per 100 cc.

	Sample 1.	Error against ash.	Remarks.
	mg.	*per cent*	
Ash................	25.9		Average of 11 determinations.
Water.............	6.2	−76.0	" " 4 "
NaCl.			
1.25 per cent.....	24.2	−6.5	" " 4 "
2.50 " "	25.6	−1.2	" " 11 "
2.50 " "	35.6	−1.2	" " 4 " + 10
			mg. Ca as $CaCl_2$.
5.00 " "	25.88	0	Average of 21 determinations.
5.00 " "	35.5	−1.2	" " 4 " + 10
			mg. Ca as $CaCl_2$.
10.00 " "	25.0	−3.4	Average of 4 determinations.

with the milk, from the sediment. Mix the milk and cream thoroughly before taking the portion for analysis.

The results obtained by the above procedure were checked against ashing in platinum. Owing to scarcity of material, only 1 cc. was used for the ash determination, titrated with 0.01 N permanganate. With this procedure the error is of the order of 2 per cent, which is the error of the titration. All determinations were made in duplicate or quadruplicate. The data given in Table I were all obtained from one large sample of milk, and show the results when water and various concentrations of sodium

chloride were added to the milk before the calcium was precipitated. Allowing the above error, correct results are obtained when the final concentration of sodium chloride is more than 1.25 per cent.

TABLE II.

Calcium Content of Human Milk.

Values expressed as mg. per 100 cc.

Sample No.	Ash.	Author's method.	Error.	Remarks.
	mg.	mg.	per cent	
2	35.0	35.6	+1.6	Mature breast milk.
3	25.6	26.6	+3.9	" " "
4	40.0	39.2	−1.9	" " "
5	23.3	23.3	0	" " "
6	51.1	51.0	−0.4	" " "
7	34.4	34.7	+0.8	" " "
8	20.6	20.4	−1.0	" " "
9	39.5	38.8	−1.8	" " " Mixed sample.
10	31.4	33.0	+5.0	" " " " "
11	20.0	17.8	−10.0	" " "
12	36.8	38.0	+3.0	Colostrum.
13	19.5	20.4	+4.0	" 3 days post partum. Centrifuged before determination.
14	18.7	19.4	+3.5	Colostrum, 4 days post partum. Centrifuged before determination.
15	38.6	37.6	−2.6	Combination of colostrum, 3 and 4 days post partum. Centrifuged before determination.
16	43.8	44.0	+0.4	Colostrum, 6 days post partum. Centrifuged before determination.
17	44.4	43.4	−2.2	Colostrum, 10 days post partum. Not centrifuged before determination.
18	22.7	22.6	−0.4	Colostrum, 8 days post partum. Not centrifuged before determination.

Table II shows the results on seventeen samples of milk, of which seven are colostrum. The maximum error in one case was −10 per cent, but as there was not sufficient material to repeat the analysis, it is not known whether the error was in the ash value or that by direct precipitation. The average error for the colostrum was +0.3 per cent, and for the normal milks, −0.2 per cent. Results are given without omission of any determinations.

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THE ELECTROMETRIC DETERMINATION OF IRON IN BLOOD.

By J. F. KING AND FREDERICK H. HOWARD.

(From the Departments of Chemistry and Physiology, Williams College, Williamstown.)

Existing methods for the quantitative determination of iron in blood are sufficiently accurate for many research purposes, and some of them, notably the rapid colorimetric methods, are now well adapted for clinical research where time together with fair accuracy is the principal consideration. There are, however, some kinds of research such as that on the physicochemical relations of hemoglobin where greater precision in the determination of iron is desirable. In the present report we are interested in describing a method adapted to the more exacting requirements.

The small percentage of iron in blood limits the total amount of iron available for analysis and so excludes from use any of the gravimetric procedures. The procedures which have been adopted for analysis are therefore either volumetric or colorimetric. There are, however, certain inherent difficulties in the reported methods which introduce errors of uncertain magnitude.

The present method which we are to describe consists in brief, first, in sampling out 5 cc. of blood directly into the flask in which the final titration is made, second, in oxidizing the organic matter by means of sulfuric acid and perchloric acid, and, third, in titrating the ferric sulfate formed by means of very dilute titanous sulfate solution, determining the end-point electrometrically. The incineration part of the process has been suggested to us by the work of Mears and Hussey (1) and the electrometric titration part is taken from the work of King and Washburne (2).

Sampling.—Defibrinated blood was pipetted with an Ostwald pipette directly into the 100 cc. long neck flask which was used for titrating. The weights of the samples of blood were obtained and

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they agreed to within 0.2 per cent of the average, an accuracy hardly obtainable with a smaller sample.

Incineration.—We used 60 per cent perchloric acid with sulfuric acid to oxidize the organic matter in place of the usual oxidizers because of the recent reports on the use of perchloric acid as an oxidizer of organic material. Mears and Hussey found in the Kjeldahl digestion of milk that the addition of 5 cc. of perchloric acid reduced the time of oxidation from $4\frac{1}{2}$ hours in the standard method to 10 minutes. Perchloric acid is a most stable acid and is known to be an excellent oxidizing agent when used in small amounts with boiling sulfuric acid. It does not decompose as easily as does chloric acid and appears to give up its oxygen gradually as the oxygen is taken up by the organic material.

The sample of blood was evaporated on a steam bath to dryness. It was then heated in an electric oven to about 150°C., well below the point at which decomposition takes place so rapidly that an iron loss results through the smoke evolved. This we consider to be one of the sources of error in the dry ashing of blood along with the loss through volatization of iron chlorides. 10 cc. of concentrated sulfuric acid were added, a trap was placed in the end of the flask, the flask was tilted at an angle, and the sulfuric acid was boiled. Boiling was continued until the sides of the flask were well cleaned down by the sulfuric acid and the sulfuric acid boiled evenly. Then the flask was cooled, 1 cc. of perchloric acid was added and the contents of the flask were again boiled. If this did not completely decolorize the mixture, the flask was again cooled and an additional few drops of perchloric acid were added. (When the reaction is complete the solution should be a pale yellow in color.) The trap was then removed from the end of the flask and the sulfuric acid boiled off into a well ventilated fume hood until there remained a residue of about 1 cc. This evaporation appeared necessary to remove all of the disintegration products of the perchloric acid which interfered with the electrometric titration. The trap was rinsed into the flask with 20 per cent sulfuric acid and enough 20 per cent sulfuric acid was added to the flask to bring the volume up to 20 to 25 cc. The flask was next heated on a steam bath until the ferric sulfate which was insoluble in the concentrated sulfuric acid had gone into solution. King and Washburne's method of electrometric titration with titanous sulfate was then used to determine the ferric iron present in the solution.

Preparation of Solutions Used.

1. *Sulfuric Acid Solution.*—20 per cent sulfuric acid was prepared by adding 325 cc. of concentrated c.p. sulfuric acid to 2400 cc. of water. The solution was boiled and hydrogen gas was passed through to remove the dissolved air. As evaporation took place, the volume was kept constant by the addition of water. The acid solution was allowed to cool in contact with hydrogen gas. An analysis of this acid showed it to have an iron content of 43 $\times$ 10^{-8} gm. per cc. This iron correction may be made in work whenever the accuracy of the analysis demands it.

2. *Standard Ferric Iron Solution.*—The standard ferric iron solution was prepared by dissolving 3.0000 gm. of clear crystals of c.p. ferrous ammonium sulfate from a freshly opened bottle in from 50 to 100 cc. of the 20 per cent sulfuric acid contained in a 500 cc. graduated flask. This solution was then titrated to the first trace of pink by a 0.2 N potassium permanganate solution (about 38 cc. required). The ferric sulfate solution was then diluted to the scratch with 20 per cent sulfuric acid. This constituted the stock ferric solution. For standardization of the titanous sulfate solution, 50 cc. of the stock solution were diluted to 500 cc., giving a solution of which 1 cc. was equivalent to 0.00008544 gm. of iron. Calibrated graduated glassware was used and the solutions were made up to volume in a thermostat bath at 20°C. Thornton and Wood (3) in a recently published report recommended the use of Bureau of Standards Sibley iron ore to standardize the titanous sulfate solutions and state that it is superior to ferrous ammonium sulfate.

3. *Titanous Sulfate Solution.*—Titanous sulfate solutions are so easily acted upon by the air that great care must be exercised to prevent the very dilute titanous sulfate solutions used in the titration from coming in contact with the air. It is desirable to prepare the standard iron solution and the titanous sulfate solution of such a concentration that with the sample of blood used, at least 20 cc. will be needed for titration. Into the air-free 20 per cent sulfuric acid solution was pipetted the correct volume of a 15 per cent stock titanous sulfate solution. This solution was then forced under an atmosphere of hydrogen into the storage reservoir of the electrometric titration apparatus by hydrogen gas from a tank.

TABLE I.

Standardization of Titanous Sulfate Solution.

Ferric iron solution.	Titanous sulfate solution.	1 cc. titanous sulfate solution equivalent to mg. iron.
cc.	*cc.*	
25	19.50	0.1095
25	19.52	0.1094
25	19.50	0.1095

TABLE II.

Analyses of Same Specimen of Blood.

5 cc. of blood used. 1 cc. of titanous sulfate solution is equivalent to 0.0001095 gm. of iron.

Titanous sulfate solution.	Iron in 100 cc. blood.	Deviation from average.
cc.	*gm.*	*per cent*
23.32	0.05107	0.24
23.24	0.05090	0.08
23.22	0.05085	0.17

TABLE III.

Second Series of Analyses.

5 cc. of blood used. 1 cc. of titanous sulfate solution is equivalent to 0.00008359 gm. of iron.

30.48	0.05096	0.02
30.47	0.05094	0.02
30.49	0.05097	0.04
30.45	0.05092	0.06

TABLE IV.

Blood iron.	Iron added.	Total iron taken.	Total iron found.	Difference.
gm.	*gm.*	*gm.*	*gm.*	*per cent*
0.002547	0.0004272	0.002974	0.002973	0.03
0.002547	0.0004272	0.002974	0.002970	0.14

EXPERIMENTAL.

Experiments were carried out on the addition of a known amount of standard iron solution to a known amount of blood iron. Typical results are given in Table IV.

25 cc. of standard iron solution were treated as the blood samples had been treated and then 5 drops of perchloric acid were added before titration. The correct amount of iron was found, showing that the perchloric acid does not oxidize the titanous sulfate solution or effect the sharpness of the end-point.

To 25 cc. of standard iron solution was added sugar and the whole was treated as the samples of blood had been, the sugar being oxidized by the perchloric acid. The correct amount of iron was found as in previous experiments.

DISCUSSION.

Certain precautions are to be emphasized.

1. Air must be completely excluded from the titrating flask during the electrometric titration.

2. The temperature of the mixture being titrated must be kept just at the boiling point. If the mixture is allowed to boil, the formation of steam bubbles on the platinum electrode interferes with the correct readings of the voltage.

3. If there is oxygen in the carbon dioxide used as a blanket over the mixture as it is being titrated, a voltage-volume curve will be obtained without the sharp drop needed to determine the end-point. In the best determinations we found the drop in voltage at the end-point to be as much as 0.150 volts for 0.1 cc. of added titanous sulfate solution. Satisfactory end-points may be obtained, however, when there is a drop in voltage of around 0.100 volt over a volume of about 0.5 cc.

4. Traces of the decomposition products of the perchloric acid give a flattened out curve at the end-point. This may be prevented by boiling off a large part of the sulfuric acid used for the digestion.

5. The gradual dissolving of the anhydrous ferric sulfate is apt to interfere with the end-point because it is possible for a slight amount, not visible to the eye, to be in suspension during the titration. This gradually goes into solution as the titanous sulfate solution is added and the result is a flattened out curve and an incorrect end-point.

When all of these possible errors have been eliminated, a voltage-volume curve, similar in shape to those shown in the article by King and Washburne, results.

SUMMARY.

We have reported a procedure for determining the amount of iron in blood with a greater precision than the methods so far published. We suggest that the procedure be used for the determination of small amounts of iron in any organic material.

Among the advantages of this method over those now in use are:

1. There is no transfer loss as the whole process is carried out in the same flask.

2. A sample of 5 cc. of blood is used but a smaller volume could be used along with more dilute solutions of titanous sulfate than those which we used. It has been shown that it is possible to realize a sharp end-point when the titration is done with a titanous sulfate solution as dilute as 0.0003 N.

3. By making use of the potentiometric end-point such disadvantages as the following are eliminated: (1) fading of the thiocyanate complex in the colorimetric method; (2) personal error involved in using the colorimeter, including the difficulty of matching slight differences in colors; (3) the difficult end-points in the volumetric processes due to the use of small volumes of solutions and the slight color changes at the end of the titration with dilute solutions.

4. By the method of plotting the results of several observations to determine the end-point, any error in a single observation tends to be averaged out.

5. Because the iron is titrated in the ferric state, errors in other oxidimetric processes, caused by the reduction part of the process, are eliminated.

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REDUCING NON-SUGARS AND TRUE SUGAR IN HUMAN BLOOD.

By MICHAEL SOMOGYI.

(From the Laboratory of the Jewish Hospital of St. Louis, St. Louis.)

It is known that the commonly used methods for blood sugar determination yield higher values than the sugar actually present. The new alkaline copper reagent of Benedict (1) and Folin's modified reagent (2), which are more specific to sugars than the older reagents, yield values still too high. In using mercury salts for the precipitation of the blood proteins, simultaneously a considerable portion of the non-sugar reducing substances is also removed, but this effect is only partial and inconsistent. As Harned (3) has shown, sugar values obtained by the Folin-Wu method in mercury filtrates are not lower than those determined by Benedict's new method in tungstic acid filtrates. Lapa (4) recently recommended precipitation by mercuric acetate as a means of obtaining values close to true sugar. His determinations were carried out only on plasma, and show 3 to 6 mg. per cent reducing non-sugars. Since the tungstic acid filtrate of plasma contains, as we shall show, not much more of these substances, Lapa's method offers no material advantage.

The older attempts to obtain true sugar values were based upon Otto's (5) original precedure of determining the "residual reduction" after alcoholic fermentation of blood, and to subtract this from the "apparent sugar." But this seemingly simple method led only to a maze of conflicting results, various authors having obtained residual reduction values ranging from zero up to 60 mg. per 100 cc. of blood, in terms of glucose. One cause of discrepancies, pointed out in the work of Frank and Brettschneider (6) and of Ege (7), is the disregard by earlier workers of the fact that various reagents, used in different reduction methods, yield different values with identical reducing substances. The main source

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of error, however, was recognized by Salkowski (8), Neuberg (9), and Mayer (10), who noted that variable amounts of reducing, and also optically active, substances are given up by yeast in the course of fermentation, even if pure glucose solutions and pure cultures of yeast were used in the experiments.

Van Slyke and his collaborators (11) have overcome this difficulty by a drastic abbreviation of the fermentation period. They, too, found that in the course of prolonged fermentation reducing substances are produced in the reaction mixture, but that this does not occur within short periods which are at the same time sufficient completely to ferment the sugar in the blood. They chose incubation for 20 minutes at body temperature as a suitable procedure. For the reduction derived from impurities introduced with the yeast they made correction.

Ege (12), taking exception to the brevity of this fermentation period, asserts on the basis of his previous experiments (7) that at least 24 hours are required for the complete fermentation of blood sugar. We have found, however, that in the course of such long fermentation periods chemical, and occasionally bacterial, changes set in, which frequently lead to an almost complete disappearance of all the non-fermentable reducing substances (13). Subsequently Folin and Svedberg (14) showed that even 8 minutes incubation with yeast suffice completely to dispose of the blood sugar.

In a preliminary note, concerning reactions between yeast and various sugars, we reported (15) the extremely rapid disappearance of glucose from solutions under certain conditions. In the course of the experiments, still in progress in this laboratory, we attempted the practical application of our finding in the determinaton of true sugar in blood (and other biological fluids) by the separation and determination of the reducing non-sugars or residual reduction. We arrived at the conclusion that it is unnecessary to incubate the blood with yeast even for the brief periods recommended by Van Slyke or by Folin and Svedberg, as the sugar is completely removed at room temperature in the course of the Folin-Wu precipitation of the blood proteins, if the water for dilution and laking is replaced by a 10 per cent (moist weight) yeast suspension.

The procedure is as follows: Into 7 volumes[1] of a 10 per cent yeast suspension introduce 1 volume of blood, thoroughly mix and agitate for a few seconds, then add 1 volume of 10 per cent sodium tungstate, mix, and finally add 1 volume of 0.66 N sulfuric acid; shake well, allow to stand for 5 minutes, and filter. Centrifugation may be inserted before filtration if small amounts of blood are used.

Preparation of Yeast.—Commercial yeast is always contaminated with adhering particles of wort which contain unfermentable reducing substances. Since the determination of these, for the purpose of correcting the reducing non-sugar values, led to inconsistent results, we decided to wash the yeast entirely free from reducing substances. This is performed as follows: A weighed amount of fresh commercial yeast (Fleischmann's) is suspended in 5 to 10 parts of water, centrifuged, and the water decanted. This operation is repeated until the supernatant liquid is practically clear and colorless, and the last washing gives a zero reduction with the copper reagent. The yeast is then suspended in 10 parts of water and is ready for use. In this condition it keeps well in the cold, especially if at intervals the water is centrifuged off and replaced by fresh water.

Apparent sugar, *mg. per cent*.......	104	116	314
Reducing non-sugars, *mg. per cent.*			
1. Precipitation performed immediately after mixing yeast and blood.....................	31	27	22
2. Precipitation 2 min. after mixing............	31	25	24
3. " 5 " " "	31	26	22
4. " 20 " " "	31		23
5. " 45 " " "		26	22

With the technique outlined we obtained consistent results in over 100 experiments with human blood. The temperature of our reaction mixtures was always that of the laboratory, 20–23°C. in most of the cases, the maximum temperature observed being 28°C. In over twenty cases we ascertained that the removal of

[1] We use 7½ instead of 7 volumes, allowing ½ volume for the cell volume of the yeast; but the error from disregarding this correction is practically negligible.

the blood sugar is complete without allowing any extra time for fermentation preceding the precipitation of proteins. A few examples—tabulated on the preceding page—will illustrate this, the rest of the experiments showing identical results.

Numerous experiments, performed in work in this laboratory along other lines, show a rapid disappearance of glucose from solutions of considerably higher concentrations than occurred in our blood samples. In the experiment given in Table I solutions containing 160, 400, and 800 mg. per cent glucose were subjected to the same procedure, including dilution and precipitation, as blood; to furnish the protein, the yeast was suspended in a neutral solution of pure casein. According to the titration figures in Table I, at a concentration of 400 mg. per cent the re-

TABLE I.

Showing the Rapid Disappearance of Glucose from Solutions in Mixture with Yeast.

Time from mixing of sugar and yeast till precipitation.	Mg. per cent glucose.		
	160	400	800
	Cc. of 0.005 N sodium thiosulfate used in titration.		
min.			
<1	22.21	22.23	21.58
2	22.22	22.22	22.02
5	22.20	22.22	22.20
20	22.21	22.19	22.23

sult is still the same whether the precipitation is performed immediately after the glucose and yeast were united, or 20 minutes later. At the extreme concentration of 800 mg. per cent, however, 5 minutes must be allowed before precipitation in order to reach a constant titration figure. The filtrate in blank experiments, in which the glucose was omitted, gave a titration figure of 22.23 cc. at the beginning of the experiment, and 22.21 cc. after the yeast suspension had been standing for 3 hours at 27.5°C. Thus the constant titration figures in Table I indicate the complete disappearance of glucose.

TABLE II.

Reducing Non-Sugars in Normal and Pathologic Human Blood.

Normal individuals.			Hospital patients.		
No.	Apparent blood sugar.	Reducing non-sugar.	No.	Apparent blood sugar.	Reducing non-sugar.
	mg. per cent	mg. per cent		mg. per cent	mg. per cent
1	100	31	34	108	31
2	76	28	72	416	29
3	108	27	401	211	29
4	94	23	404	144	24
5	102	29	405	113	26
6	99	28	538	100	23
7	120	28	544	87	25
8	107	27	545	101	25
9	108	26	548	109	29
10	110	31	549	175	27
11	120	25	550	103	26
12	113	28	551	226	28
13	136	25	556	234	26
14	124	28	559	90	25
15	94	30	561	175	23
16	147	25	010	108	27
17	100	27	018	174	25
18	112	25	019	82	23
19	96	25	020	113	28
20	97	23	021	215	30
21	163	28	029	214	22
22	116	26	030	89	27
23	150	29	031	133	24
24	137	29	034	224	29
25	123	27	035	244	30
26	110	24	084	99	26
Lowest.................		23			23
Highest............ ..		31			31
Average................		27			26

Reducing Non-Sugars in Health and Disease.

We have determined the reducing non-sugars in the blood of twenty-six healthy individuals and in a large number of samples from hospital patients. Of these twenty-six are given in Table

II, picked in the chronological order as they were examined, with the exclusion only of samples showing high nitrogen retention.

The values in this work were obtained by the Shaffer-Hartmann method, with the reagent modified by the writer (16). An interesting fact disclosed in this table is the remarkable uniformity of the amount of reducing non-sugars: both in health and in disease it is 23 to 31, an average of 27 mg. in 100 cc. of blood, expressed in terms of glucose. The only exceptions to this were cases with high nitrogen retention, in most of which—as shown in Table III—the amount of reducing non-sugars rises above the normal level. This is in line with the findings of Hiller, Linder, and Van Slyke (11) who reported residual reduction values of 40

TABLE III.
Reducing Non-Sugars in Nitrogen Retention.

Case No.	Apparent sugar.	Reducing non-sugar.	Non-protein nitrogen.
		Mg. per 100 cc.	
517	109	36	88
555	312	31	82
298		28	68
303		41	185
387	123	43	160
403	119	38	60
404	144	24	56
405	113	26	44
418	297	36	71

to 48 mg. per cent (Folin-Wu method) in the fermented blood of patients with glomerulonephritis. There is, however, no direct relation between non-protein nitrogen and reducing non-sugars, and even normal values for the former are compatible with high non-protein nitrogen.

The other important characteristic of the reducing non-sugars, as shown in Table II, is their independence of the blood sugar level. Previous workers found greatly increased residual reduction in various cases of hyperglycemia, such as diabetic and hemorrhagic hyperglycemia (5, 17). Lund and Wolf (18) recently determined in diabetic blood as much as 36 to 91 mg. per cent

of unfermentable reducing substances. Among the specimens in our experiments some were drawn from fasting individuals, some in the state of alimentary hyperglycemia, and a few after the subject had given 500 to 600 cc. of blood for transfusion; but the reducing non-sugars are unaffected by these factors. The samples from hospital patients comprise a number of diabetic bloods which exhibit, without exception, a normal amount of reducing non-sugars. The lack of parallelism between variations of the sugar level and the reducing non-sugars—as also pointed out by Folin and Svedberg (14)—is still more accentuated in a few experiments

TABLE IV.

Relation between Reducing Non-Sugars and Changes in Blood Sugar Level.

Time after ingestion of glucose.	Case 523.		Case 023.		Case 048.	
	Apparent sugar.	Reducing non-sugar.	Apparent sugar.	Reducing non-sugar.	Apparent sugar.	Reducing non-sugar.
	Mg. per 100 cc.					
min.						
0 (Fasting.)	109	28			272	27
30			355	33	375	
60	183	30	414	28	410	
120	142	30	433	29	459	23
180	77	28	447	26	465	23

given in Table IV, in which the amount of reducing non-sugars was followed up in sugar tolerance tests, after the ingestion of 100 gm. of glucose. While in none of the three cases are the reducing non-sugars increased with rising blood sugar, in the severe diabetic cases, Nos. 023 and 048, there is a distinct decline as the high sugar level persists. Van Slyke and his associates (11) found that the residual reduction is likewise unaffected by insulin hypoglycemia in rabbits even though the blood sugar be reduced to zero in insulin convulsions.

True Sugar Values.

Before concluding the difference between apparent sugar and reducing non-sugars to be true sugar, we had to consider another possible source of error. According to Holden (19) amino acids,

as glycine, cystine, glutamic acid, giving alone little or no reduction with alkaline copper solutions, appreciably increase the reduction values of added glucose. We had to ascertain, therefore, whether or not similar induced or coupled reactions occur in the determination of apparent sugar; in other words, whether the apparent sugar represents the actual sum of true sugar and reducing non-sugars or perhaps some higher value. To this end we prepared sugar-free blood filtrates and determined their reduction values with and without the addition of known amounts of glucose. The result invariably was the sum of the reduction exerted by the non-sugars plus the reduction calculated for the added glucose. For example:

With 5 cc. of copper reagent were heated for 15 minutes in a water bath 3 cc. of sugar-free filtrate of corpuscles + 2 cc. of H_2O; reduction = 23 mg. per cent in terms of glucose.

3 cc. of H_2O + 2 cc. of glucose solution; reduction = 96 mg. per cent glucose.

3 cc. of sugar-free filtrate of corpuscles + 2 cc. of glucose solution; reduction = 118 mg. per cent in terms of glucose.

23+96 = 119.

Thus the difference between apparent sugar and reducing non-sugars is the true sugar. In most cases it is not necessary to determine the exact amount of the reducing non-sugars; because of the uniformity of this value, it is sufficiently accurate for most purposes to use 27 mg. per cent as a correction. By subtracting this amount from the apparent sugar, the true sugar is obtained with a maximum error of $\pm$ 4 mg. per cent. The probable error is even less, as reducing non-sugar values in excess of 29 mg. per cent and below 24 mg. per cent are relatively infrequent.

Similar corrections may be readily established for any other method for blood sugar determination, the above figure applying only to the Shaffer-Hartmann method.

Distribution and Chemical Nature of Reducing Non-Sugars.

In previous, unpublished experiments (carried out in the Department of Biochemistry, Washington University School of Medicine) we have found that Folin-Wu filtrates and mercury filtrates of beef plasma yield practically the same sugar values, whereas from corpuscles, 50 to 70 mg. per cent lower values are

obtained in mercury filtrates than in tungstic acid filtrates. This finding prompted an examination of the distribution of reducing non-sugars in corpuscles and plasma of human blood. Twenty specimens, obtained from healthy persons, were analyzed with the following result.

Reducing Non-Sugars, Mg. per 100 Cc.

	In whole blood.	In plasma.	In corpuscles.
Lowest	23	7	41
Highest	31	13	51
Average	27	10	47

These figures show that corpuscles contain, on an average, about 5 times as much reducing non-sugars as plasma. Rockwood (20) too, found that a substance, oxidized by the Folin-Wu sugar reagent but unaffected by Benedict's new reagent, is chiefly confined to the corpuscles.

We concur in the opinion of Rockwood and of Sjollema (21) that only a small fraction of the reducing non-sugars is represented by those constituents of Folin-Wu filtrates which are known to reduce alkaline copper solutions. Van Slyke (11) has demonstrated that in normal blood the sum of the reduction values of uric acid and creatinine does not exceed the equivalent of 2 to 3 mg. per cent of glucose, and only in cases of high nitrogen retention does it rise to 10 to 15 mg. per cent. This increase closely corresponds to the excess over the normal values in the specimens from nephritic patients given in our Table III. Thus it is safe to assume that some other substance or substances, as yet unidentified, are responsible for the greater part of the reduction derived from non-sugars.

The fact that the distribution of the reducing non-sugar substances in corpuscles and plasma coincides so conspicuously with that of Benedict's (22) thioneine or the substance X of Hunter and Eagles (23), renders it highly probable that this interesting sulfur compound—shown to be identical with ergothioneine (24–26)—has a part in the reduction exerted by tungstic acid filtrates. By the courtesy of Dr. Eagles we obtained a sample of ergothioneine and performed the following experiments:

With 5 cc. of copper reagent were heated for 15 minutes in a water bath 3 cc. of 10 mg. per cent ergothioneine + 2 cc. of H_2O; reduction value = 23 mg. per cent in terms of glucose.

3 cc. of H_2O + 2 cc. of glucose solution; reduction value = 95 mg. per cent glucose.

3 cc. of 10 mg. per cent ergothioneine + 2 cc. of glucose solution; reduction value = 116 mg. per cent in terms of glucose.

95+23 = 118.

Results of the same nature were obtained with other concentrations of ergothioneine and of glucose. As can be seen, ergothioneine in a concentration at least as high as the maximum found in corpuscles, yields only a reduction equivalent to 23 mg. per cent of glucose so that an additional non-sugar must account for the high reducing non-sugar content of corpuscles. Thompson and Voegtlin (27) found that glutathione is present only in the corpuscles, furthermore that it is largely in the reduced form. Hunter and Eagles (28) estimate the amount of glutathione in human and animal corpuscles as 100 mg. per 100 cc. and concur in the opinion that it is, at least for the most part, in the reduced form. Thus, we believe that glutathione is responsible for a substantial fraction of the residual reduction in tungstic acid filtrates of corpuscles.

This contention is apparently in contradiction to the fact that thioneine does not reduce alkaline copper solutions (22), and that probably the same holds true for glutathione, at least in its oxidized form. But it must be borne in mind that glutathione as well as thioneine consumes iodine even in the cold so that in our method, and at least in all of those which conclude in iodometric titration, their presence increases the reduction values irrespective of their reducing effect upon the copper reagent. In fact, tungstic acid filtrates of blood consume iodine at room temperature in acid medium, although somewhat less than the equivalent of the reducing non-sugars. This iodine consumption is the same whether or not the sugar is removed from the filtrate.

SUMMARY.

A method is presented for the determination of reducing non-sugars (residual reduction) and thereby for the estimation of true sugar in blood.

The amount of reducing non-sugars in human blood is found to be very uniform, averaging 27 mg. per 100 cc. of blood in terms of glucose, as determined by the Shaffer-Hartmann method with

the modified reagent. It is independent of the blood sugar level, and rises above the normal only in cases of high nitrogen retention.

The distribution of reducing non-sugars in corpuscles and plasma is unequal; the average value for corpuscles is 47 mg. per 100 cc., for plasma 10 mg. per 100 cc.

In human blood the subtraction of 27 mg. per cent from the apparent sugar, as determined with the modified Shaffer-Hartmann reagent, gives the true sugar with a maximum error of ± 4 mg. per cent.

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OBSERVATIONS ON THE METABOLISM OF DIHYDROXY-ACETONE IN NORMAL AND DIABETIC INDIVIDUALS.

By I. M. RABINOWITCH.

(From the Department of Metabolism, the Montreal General Hospital, Montreal, Canada.)

In a preliminary report (1) it was shown that diabetics were apparently able to tolerate dihydroxyacetone more readily than glucose. In the same individual the character of the blood sugar time curve following ingestion of this triose differed from that obtained after glucose. With dihydroxyacetone, the maximum increment in the blood sugar concentration was less, and the rate of decline of the curve was more rapid. In diabetics excreting sugar the rates of excretion of the latter appeared to diminish after ingestion of this triose. No evidence of renal damage was found to account for the latter phenomenon. Though these were short period experiments, subsequent observations proved that this was not the explanation of the results obtained. During further studies (2) it was found that when dihydroxyacetone was administered to normal individuals and diabetics, in amounts not greater than the determined rates of utilization of glucose (respiratory quotient studies), the concentration of total sugar in the blood not only did not increase, but was found to decrease. Observations on the respiratory exchange appeared to indicate that the rate of utilization of dihydroxyacetone was greater than that of glucose both in normal and diabetic subjects. In one diabetic with marked ketosis (3) there appeared to be evidence that dihydroxyacetone exerted an antiketogenic action. From studies upon diabetics under very careful dietary control, it was suggested that it may be possible to dispense with small doses of insulin without reduction in the total caloric value of diets, by substituting dihydroxyacetone for part of the other carbohydrates. In view of past literature and the above observations it was also

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suggested that dihydroxyacetone was probably in the main path of intermediate metabolism of carbohydrates, and could be oxidized directly without preliminary conversion into glucose.

Shortly after, in a series of reports (4–7) Campbell and his coworkers recorded their observations on this sugar, and their findings were diametrically opposite to ours. These workers concluded from their experiments that dihydroxyacetone must be converted into glucose before its utilization, and therefore the possibility is precluded of this triose possessing a greater clinical value in diabetes than other substances, such as glycerol, which are more slowly converted into glucose than the starches.

Our immediate interest in the metabolism of dihydroxyacetone was the possibility of its use in the treatment of diabetes. If it were found that this sugar possesses antiketogenic properties and the diabetic could utilize it, even in small quantities, when the limit of tolerance of glucose had been reached, it might be possible, at least in border-line cases, by substituting it for other carbohydrates, to dispense with small doses of insulin, which must be administered subcutaneously. Since in the majority of cases the diabetes is not very severe, the wide application of a sugar with such properties becomes obvious. It might also, if rapidly oxidized, be of value in diabetic coma when combined with insulin.

Because of the foregoing observations, one important fact which must be established is whether or not dihydroxyacetone must, as stated by the Toronto workers, be first converted into glucose before it can be utilized. Since the majority of our experiments was not similar to those reported by the Toronto workers, it was hardly possible to compare data. An analysis of the results of these workers, however, led to certain observations, and it was considered advisable, in order to avoid polemics, to accumulate more information, based upon experiment only, before recording them. This we have done, and the purpose of this paper is to record our findings. These appear to demonstrate that there is a difference between the utilization of glucose and dihydroxyacetone. In the meantime, however, other laboratories have taken up this work, and the results of the greater part of our preliminary experiments have, on the whole, been confirmed (8–12).

Results similar to ours were first reported by Mason (8) who

found that, in hospital patients with normal carbohydrate metabolism, the ingestion of dihydroxyacetone resulted in a very slight and transitory rise of the blood sugar. *When given in small doses there was a progressive fall.* In diabetics the increment in blood sugar was not as great after dihydroxyacetone as after a like dose of glucose. When given intravenously to diabetics with fair carbohydrate tolerance in 25 gm. doses, little increases of the blood sugars were noted. When ingested in small, but frequent doses, the dihydroxyacetone so administered could not be accounted for by the urinary excretion of glucose nor by tissue retention. Respiratory exchange studies showed that dihydroxyacetone caused a more rapid and greater carbohydrate combustion than similar doses of glucose given to the same individual. Fourteen experiments on seven patients including mild and moderately severe diabetics, indicated that the respiratory metabolism of this triose was fundamentally different from that of glucose. The average increment of the non-protein respiratory quotient after glucose was 0.048, whereas after dihydroxyacetone it was 0.130. These findings agreed very closely with those of Himwich, Rose, and Malev (11), who found that, in dogs, *in contradistinction to glucose*, subcutaneous administration of dihydroxyacetone caused an immediate and considerable rise in the respiratory quotient. During the first 15 minutes of the experiments the average increase of the latter, due to glucose, was 0.04, whereas that due to dihydroxyacetone was 0.19. These authors suggested that, unless the rates of absorption of glucose and dihydroxyacetone were different, their experiments indicated that dihydroxyacetone was more readily available than glucose when administered subcutaneously.

Results obtained in the Edinburgh laboratories by Kermack, Lambie, and Slater (12), in experiments similar to our own were practically identical with ours. These authors record a rather striking case. A pregnant diabetic on a constant diet and 20 units of insulin was excreting 1 gm. of glucose *per diem* and acetone. With 15 units of insulin the excretion of sugar increased to 10 gm., thus indicating the necessity of the 20 units of insulin. With 20 units of insulin and the same diet it was possible to add 30 gm. of dihydroxyacetone, with practically no increase in the excretion of sugar (2 gm. *per diem*). The acetone bodies disap-

peared.   In discussing the results of the Toronto group of workers, more of the findings of the Edinburgh workers will be referred to.

In their reports, (4, 6, 7) the Toronto workers emphasize certain ideas particularly.   Firstly (4) they regard it as very doubtful that dihydroxyacetone is in the main path of intermediary metabolism of carbohydrates, and one of the arguments presented in favor of this view (5) is that dihydroxyacetone is not found in blood of normal individuals, but is found in diabetics in *large amounts, according to their method of its determination,* (italics ours) after its ingestion.   This will be referred to in the subsequent discussion on technique.   Another of their ideas is that proof of the necessary preliminary conversion of dihydroxyacetone into glucose before its utilization is presented in the results obtained (6) with its use in insulin hypoglycemia.   It is interesting here to note that though Kermack, Lambie, and Slater (12) were also able to relieve the symptoms of insulin hypoglycemia with dihydroxyacetone, they found, *by experiment,* that this could not have been due to the conversion of the dihydroxyacetone administered into glucose.   A very appropriate type of experiment was reported. After excluding the liver and kidneys from the circulation of a cat, the resultant tendency of the blood sugar to fall was counterbalanced by transfusion of glucose.   Dihydroxyacetone was then substituted at the same uniform rate as that of the glucose. During this period the blood sugar fell, and at the same time no significant change had taken place in the concentration of dihydroxyacetone in the blood.   This result indicates that the dihydroxyacetone injected was almost immediately utilized, and at a much greater rate than the glucose.   A significant finding was that during the dihydroxyacetone administration the total utilization of carbohydrates was increased; for not only was the dihydroxyacetone completely used up, but also a considerable amount of glucose disappeared from the blood.   It is interesting here to note that, in our preliminary report (1), it was also shown that following ingestion of dihydroxyacetone the excretion of sugar in the urine of diabetics diminished, and this could not be accounted for by impairment of kidney efficiency.   On reviewing all their work, Kermack, Lambie, and Slater conclude that all the phenomena observed are readily explained on the simple assumption that dihydroxyacetone is very easily and possibly directly oxidized and utilized by the animal organism.

Experimental proof presented by the Toronto workers of the necessary conversion of dihydroxyacetone into glucose before it can be utilized was the report of the identity of effects of the administration of glucose and dihydroxyacetone on the inorganic phosphorus contents of the bloods of normal individuals (7).

TABLE I.

Data of Inorganic Blood Phosphorus Time Curves of Normal Individuals Following Ingestion of 100 Gm. of Glucose.

The values are expressed in mg. of inorganic phosphorus per 100 cc. of blood.

Subject No.	Period.				
	Fasting.	30 min.	60 min.	120 min.	180 min.
1	3.14	3.02	3.00	2.71	2.63
2	2.50	2.51	2.12	2.00	1.76
3	3.31	2.92	2.63	2.15	1.96
4	2.71	2.82	2.50	2.20	2.42
5	3.69	3.41	3.07	2.90	2.86

TABLE II.

Data of Inorganic Blood Phosphorus Time Curves of Diabetics Following Administration of Insulin.

The values are expressed in mg. of inorganic phosphorus per 100 cc. of blood.

Subject No.	Period.				
	Fasting.	30 min.	60 min.	120 min.	180 min.
1	3.70	3.20	3.00	3.02	3.20
2	3.41	3.00	2.60	2.60	2.74
3	3.20	3.01	2.72	2.53	2.41
4	2.61	2.50	2.50	2.41	2.32
5	2.45	2.01	1.47	1.86	2.26

The detailed data of their experiments are not recorded. It is rather difficult, however, to find the applicability of this finding. If phosphates are essential for the metabolism of carbohydrates and this is now generally accepted, and if both sugars are utilized by the normal individual, one would, *a priori*, expect such results.

The important experiment, which the Toronto workers appear to have overlooked, is that which would determine whether the blood phosphates of a diabetic would be lowered following dihydroxyacetone administration when no lowering occurred after giving glucose. We have paid particular attention to this phase of the metabolism of dihydroxyacetone and the following are the data of a series of observations made.

The technical methods employed for the estimation of blood

TABLE III.

Data of Comparative Blood Phosphorus Time Curves of Normal Individuals Following Ingestion of Glucose and of Dihydroxyacetone.

The values are expressed in mg. of inorganic phosphorus per 100 cc. of blood.

Subject No.	Sugar.	Period.				
		Fasting.	30 min.	60 min.	120 min.	180 min.
1	Glucose	4.12	3.86	3.91	3.78	3.80
	Dihydroxyacetone.	3.85	3.30	3.26	3.42	3.65
2	Glucose.	3.62	3.30	2 97	3.04	3.12
	Dihydroxyacetone.	3.12	2.54	2.50	2.26	2.96
3	Glucose.	3.78	3.65	3.42	3.67	3.70
	Dihydroxyacetone.	3.27	3.01	2.87	3.05	3.30
4	Glucose.	3.50	3.31	3.19	2.65	3.08
	Dihydroxyacetone.	4.14	4.09	3.67	4.20	4.13
5	Glucose.	2.97	2.62	2.05	3.04	3.15
	Dihydroxyacetone.	3.05	2.42	2.56	3.11	3.18

and urinary sugars were previously described (1). For the estimation of blood and urinary phosphates, Briggs' modification (13) of the Bell-Doisy method was followed.

In normal indivduals following the ingestion of carbohydrates the concentration of the inorganic phosphates of the blood decrease. This is generally recognized. In diabetics the same is noted following administration of insulin. The magnitudes of such decreases of the blood phosphates may be seen in the data of Tables I and II. Each of the five normal subjects was given 100

gm. of glucose. Each of the five diabetics received 25 units of insulin. All tests were commenced in the fasting state, and observations were then made at the end of 30 minutes, 1, 2, and 3 hours.

Comparative blood phosphate time curves were obtained following administration of dihydroxyacetone and glucose in normal and diabetic individuals. In Table III are shown the results in five normal subjects. There appear to be no great

TABLE IV.

Data of Comparative Blood Phosphorus Time Curves of Diabetics Following Ingestion of Glucose and of Dihydroxyacetone.

The values are expressed in mg. of inorganic phosphorus per 100 cc. of blood.

Subject No.	Sugar.	Period.				
		Fasting.	30 min.	60 min.	120 min.	180 min.
1	Glucose.	3.07	3.06	3.11	3.09	3.17
	Dihydroxyacetone.	3.18	2.79	2.81	3.20	3.18
2	Glucose.	4.12	4.08	4.15	4.19	4.07
	Dihydroxyacetone.	3.86	3.58	3.52	3.92	3.86
3	Glucose.	3.20	3.25	3.22	3.21	3.17
	Dihydroxyacetone.	3.41	3.16	3.02	3.29	3.38
4	Glucose.	2.86	2.92	2.89	2.80	2.84
	Dihydroxyacetone.	3.01	2.64	2.72	3.11	3.16
5	Glucose.	3.89	3.34	3.42	3.90	3.88
	Dihydroxyacetone.	3.67	3.01	3.38	3.74	3.81

differences between the effects of both sugars in normal individuals, except that the depression of the phosphate values appears, on the average, to be more marked when dihydroxyacetone was given.

When glucose was given to diabetics, in 25 gm. doses, no appreciable effects were noted on the phosphate values in four of the five instances. When, however, dihydroxyacetone was given in like doses to the same subjects the days following the administration of the glucose, definite depressions of the blood phosphate

values were noted in all of the five subjects.   The results of these experiments are shown in Table IV.

In all the five cases the diabetes was regarded as severe.   The selection of such cases was so made in order to make reasonably certain that little or none of the glucose given would be utilized (oxidized or stored).   It will be noted that the selection failed in one case (Subject 5).

In view of the above findings it would appear that the diabetic is able to utilize dihydroxyacetone in spite of the intolerance towards glucose.   These results appear to be significant in view of the fact that the dihydroxyacetone was, in each case, given on the day *following* the glucose administration.   Though in a normal individual ingestion of glucose is supposed to lead to stimulation of the glycogenic function, the administration of glucose to a diabetic is the chief cause of deterioration of this function (Allen).   One would therefore hardly expect these diabetics, who could not utilize glucose, to be able to utilize the dihydroxyacetone the following day, if the utilization of the latter depended upon its preliminary conversion into glucose.

It is obvious that greater significance could be attached to such experiments if the rates of utilization of carbohydrates (determined by the respiratory metabolism) had been determined simultaneously.   During the last few years we have followed a technique for such observations.   This has been described elsewhere (14); no repetition here is therefore necessary.   This technique has been applied in this study.   It was thus possible to obtain, simultaneously, data with reference to the following: blood sugar, blood phosphates, urine sugar, urine phosphates, and respiratory quotients.

The results of one such experiment are shown in Table V.   In this case on 3 alternate days, glucose, dihydroxyacetone, and dihydroxyacetone phosphate respectively were administered in 25 gm. doses.[1]

It will be noted that, in contradistinction to glucose, the administration of dihydroxyacetone was followed by a decrease in the concentration of blood phosphates, a decreased rate of urinary

excretion of phosphates, and an increase of respiratory quotient. Here again we note a fall of the blood sugar and decreased excretion of sugar following dihydroxyacetone. It is interesting to note that though dihydroxyacetone phosphate was also apparently

TABLE V.

Data of Simultaneously Determined Blood Sugar, Blood Phosphorus, Urine Sugar, Urine Phosphorus, and Respiratory Quotient Time Curves of a Diabetic Following Ingestion of Glucose, Dihydroxyacetone, and Dihydroxyacetone Phosphate.

Determination.	Sugar.	Period.				
		Fasting.	30 min.	60 min.	120 min.	180 min.
Blood sugar, *per cent.*	Glucose.	0.181	0.230	0.214	0.200	0.208
	Dihydroxyacetone.	0.206	0.184	0.164	0.149	0.152
	Dihydroxyacetone phosphate.	0.174	0.170	0.169	0.146	0.160
Blood phosphates, *mg. per 100 cc.*	Glucose.	3.55	3.52	3.60	3.51	3.48
	Dihydroxyacetone.	3.64	3.32	2.14	2.08	2.12
	Dihydroxyacetone phosphate.	3.59	3.60	3.57	3.62	3.41
Urine sugar, *gm. per hr.*	Glucose.	1.74		2.51	2.42	2.03
	Dihydroxyacetone.	2.16		1.34	1.31	0.97
	Dihydroxyacetone phosphate.	1.85		1.01	0.85	0.68
Urine phosphates, *mg. per hr.*	Glucose.	37.5		36.2	42.0	38.4
	Dihydroxyacetone.	40.2		24.1	26.2	20.3
	Dihydroxyacetone phosphate.	42.7		43.6	36.4	38.1
Respiratory quotients.	Glucose.	0.724		0.727	0.734	0.730
	Dihydroxyacetone.	0.714		0.798	0.784	0.786
	Dihydroxyacetone phosphate.	0.735		0.771	0.780	0.776

oxidized and influenced the excretion of glucose, it had no appreciable effect upon the blood or urine phosphates.

The rise in the R.Q. following administration of dihydroxyacetone in this case can hardly be explained on the basis of mild

diabetes. This is suggested in view of the response to glucose ingestion by the same individual.

An unexpected finding and one difficult to explain was a rise of the respiratory quotient accompanied by a *decrease* of the blood phosphates. One would expect that as oxidation took place the phosphorus would be released and would result in an increased rather than a decreased concentration of blood and urinary phosphates. No explanation of this is offered.

A rather remarkable finding by the Toronto group of workers and difficult to explain was that in all their cases recorded (5), with one exception, in which glucose and dihydroxyacetone were given in like doses on separate days to normal individuals, dihydroxyacetone caused a higher rise in the total blood sugar than did glucose. Thus:

Maximum Concentration of Total Blood Sugar (Gm. per Liter).

Subject.	After glucose	After dihydroxyacetone.
Pn	1.38	1.64
F	1.27	1.34
Pk	1.11	1.49
D	1.19	1.04

We have performed this type of experiment many times both in normal and diabetic subjects and have yet to encounter this result. It may be observed that Mason's findings (8) and those of Kermack, Lambie, and Slater (12) are identical with ours in this respect.

In contradistinction to our experience and that of Kermack, Lambie, and Slater, the Toronto workers also record marked increases in the concentrations of dihydroxyacetone of bloods of diabetics after ingestion of this triose. A possible explanation of this was sought in the technical procedures employed. Campbell's method for the determination of dihydroxyacetone (5) is based upon the fact, previously noted by Miller and Taylor (15), that this triose will under a certain set of conditions reduce molybdate solutions. It is a colorimetric method, and the following observations appear relevant.

Colorimetric methods to be reliable, for quantitative purposes, should conform to Beer's law; that is, the intensity of color should vary directly as the concentration of the substance measured.

The substance responsible for the blue color produced by the reaction between dihydroxyacetone and the Folin-Wu phosphate molybdate reagent is of such a nature that, with dilution, its solutions not only do not obey Beer's law but actually *change in shade*. Campbell recognizes this by stating that: "The unknown should not be less than three-fourths, nor more than one and one-half times the strength of the standard, as the colors do not exactly match if these limits are exceeded." The precautions suggested by Campbell do not alter the fact that, because of this peculiar action, namely change in shade (probably an ionization phenomenon), the method is not strictly applicable for quantitative work. That it is not quantitative becomes more obvious from the following observations.

It appears logical to assume that, if a test can be found sufficiently delicate to detect dihydroxyacetone in blood filtrates, in concentrations much less than those Campbell claims to have found by his method, and if by this method dihydroxyacetone is not detected after ingestion of dihydroxyacetone in amounts Campbell and his coworkers employed, the applicability of their method must be questioned.

A simple technique for the qualitative detection of dihydroxyacetone is as follows: To 2 cc. of the Folin-Wu blood filtrate are added 2 cc. of the Folin-Wu copper reagent. This is allowed to stand for $\frac{1}{2}$ hour *at room temperature*. Following this, 2 cc. of the Folin-Wu phosphate molybdate solution are added. This is allowed to remain at room temperature for 5 or 10 minutes, and the color developed is compared with the similarly treated blood filtrate which is obtained before the administration of dihydroxyacetone. Observations are made by looking down upon the column of liquid in the test-tubes. These tests are performed in what are generally recognized as Wassermann test-tubes (100 × 10 mm.). Thus for this quantity of solution (6 cc.), a column of liquid 60 to 70 mm. in length is obtained, and at the same time it has a very small surface, thus minimizing *the possibility of re-oxidation of the copper reagent in the time required*. If serial dilutions of watery solutions of dihydroxyacetone are made for comparison, the test becomes, at least approximately, quantitative.

To test the sensitivity of this method different amounts of dihydroxyacetone were added to water, and also to Folin-Wu

blood filtrates, and thus solutions of different concentrations were prepared.   By this procedure it can be very readily demonstrated that dihydroxyacetone can be detected in concentrations of as little as 1 mg. per 100 cc. of either watery solution or of blood filtrate.   If the final mixture is allowed to remain for a greater length of time (12 hours or so), it is possible to detect this sugar in a concentration of as little as 0.1 mg. per 100 cc. of liquid.

In the four blood sugar time curves (see Table V (5)), which Campbell obtained, the concentrations of dihydroxyacetone at the 30 minute period were as follows, expressed as mg. per 100 cc. of blood.

ьject.	Dihydroxy-acetone.
Pn	70
F	29
Pk	56
D	41

In order further to test our method we prepared watery solutions of dihydroxyacetone in concentrations corresponding to one-tenth of the above values, since Folin-Wu blood filtrates represent one in ten dilutions of blood.   In no case, as was to be expected, did we fail to detect dihydroxyacetone in these solutions by this method.   Blood filtrates of normal individuals and also of diabetics were then also treated with dihydroxyacetone in order to simulate the concentrations of filtrates found by Campbell.   In no case did we fail to detect dihydroxyacetone in any of these solutions.

In order, however, to determine definitely whether dihydroxyacetone appears in the blood of diabetics in large quantities after its ingestion, four diabetics were given 100 gm. of this sugar, and 30 minutes later the bloods were examined by Campbell's method, that described by the Edinburgh workers, and also by our procedure described above.[2]   The results are shown in Table VI. By both the method of the Edinburgh workers and our method were we unable to detect appreciable quantities of dihydroxyacetone, but we did find quantities by Campbell's method in amounts

[2] By preparing solutions of different concentrations of dihydroxyacetone for comparative purposes our method becomes approximately quantitative.

varying from 32 to 85 mg. per 100 cc. of blood. The sensitivity of the reaction, which we described above, has been tested so frequently that we can hardly help but suggest that the method employed by the Toronto workers may explain some of their results.

The fact that dihydroxyacetone can be converted into glucose requires no discussion. Though the demonstration of this phenomenon forms a great part of the subject matter in three of the four publications of the Toronto group of workers, one can hardly find its purpose. This characteristic of dihydroxyacetone, and in fact of all the trioses, has been recognized for years. It was referred to in our preliminary report, and is referred to in practi-

TABLE VI.

Concentrations of Dihydroxyacetone of Bloods of Diabetics after Ingestion of 100 Gm. of Dihydroxyacetone.

The values are expressed in mg. per 100 cc. of blood.

Subject No.	Method.		
	Campbell.	Kermack et al.	Author's.
1	42	16	Less than 10.
2	56	8	" " 10.
3	35	14	" " 5.
4	85	9	" " 10.
5	71	11	" " 5.

cally all works on the intermediary metabolism of carbohydrates. Given in *excessive quantities,* particularly by mouth, conversion of dihydroxyacetone into glucose is most likely to occur. We have previously suggested (2) an explanation of this. The medium of the intestinal canal is alkaline, and one of the well known characteristics of dihydroxyacetone is the readiness with which it can be polymerized in even slightly alkaline media.

The following experiments have also been performed, the results of which appear to be difficult to explain except on the basis that there is a difference between the utilization of dihydroxyacetone and glucose.

Five diabetics were selected and on different days they received glucose and dihydroxyacetone. Simultaneous arterial and venous

blood determinations were made following ingestion of these sugars. The value of such studies in diabetes was first demonstrated by Lawrence (16), and subsequently in a relatively large series of cases in our laboratories (17). It appears reasonable that in the absence of utilization (oxidation or storage) of carbohydrates, the concentration of sugar in blood leaving any particular mass of body tissue will be the same as that of the blood enter-

TABLE VII.

Data of Simultaneoulsy Determined Arterial and Venous Blood Sugar Time Curves of Diabetics Following Ingestion of Glucose and of Dihydroxyacetone.

The values are expressed in per cent.

Subject No.	Sugar.	Fasting.	30 min. later.		
		Venous blood.	Arterial (A).	Venous (V).	A − V
1	Glucose.	0.143	0.270	0.263	0.007
	Dihydroxyacetone.	0.140	0.204	0.181	0.023
2	Glucose.	0.166	0.256	0.250	0.016
	Dihydroxyacetone.	0.172	0.232	0.198	0.034
3	Glucose.	0.250	0.322	0.320	0.002
	Dihydroxyacetone.	0.263	0.302	0.285	0.017
4	Glucose.	0.222	0.285	0.286	0.001
	Dihydroxyacetone.	0.200	0.202	0.178	0.024
5	Glucose.	0.149	0.232	0.217	0.015
	Dihydroxyacetone.	0.143	0.192	0.151	0.041

ing. It has been shown (16, 17) that the more severe the diabetic the less the difference between the arterial and venous blood sugar contents after glucose ingestion.

In Table VII are shown the results of these experiments. In each case 25 gm. of glucose were given in the fasting state, and the arterial and venous blood sugar contents determined 1 hour later. The following day the tests were repeated, the same doses of dihydroxyacetone being employed. No arterial blood deter-

minations were made before the ingestion of glucose, since it has been demonstrated (16, 17), that regardless of the degree of hyperglycemia there are very little arteriovenous differences (except in coma).

It will be noted that arteriovenous differences were greater after dihydroxyacetone administration than after glucose. One would hardly expect these findings if dihydroxyacetone must be converted into glucose prior to its utilization.

In their discussion on the metabolism of carbohydrates the Toronto workers refer, in support of their views, to the observation of one of their workers (18) that the action of insulin on carbohydrates is of the monomolecular type. Attractive and interesting as this idea may be, one must recognize the fact that this has not by any means been proven to the extent of being applicable here. One may here recall numerous experiences of the past with other biological phenomena, at first purported to have been monomolecular reactions. Apropos of applying mathematical equations to chemical reactions, it may not be out of place to repeat the observation that "the student must not fall into the trap of believing that because observed and calculated results agree, therefore the theory in question necessarily applies. For whilst it is the case that disagreement between the observed and calculated results is definite evidence against the theory in question, agreement between the two results is not absolute evidence in its favor." A biological phenomenon, such as the reaction of the individual to insulin, is complicated to such an extent by simultaneous side and secondary reactions that a specific coefficient of velocity can—at least with the experimental methods at present available—be calculated only with the greatest difficulty, if at all.

We believe we have, in the above data, evidence that the metabolism of dihydroxyacetone differs from that of glucose and that for the utilization of this triose preliminary conversion into glucose is not necessary. Theories based upon and explanations of experimental data tend to change with accumulation of more experience. Experimental data should, however, be incontestable. The data are recorded in detail. The experiments are simple and may readily be repeated in any well conducted laboratory.

Addendum.—Since the above paper was submitted for publication, another paper on the same subject by Lambie and Redhead of Edinburgh University has appeared in the *Biochemical Journal* (19). Included in the work of the latter authors there are blood sugar, blood phosphate, and respiratory metabolism time curves. The results obtained were practically identical with ours. The work of these authors, as our own, does not confirm the observations of Campbell, Fletcher, Hepburn, and Markowitz, regarding the identical behavior of the inorganic blood phosphate curves after administration of glucose and dihydroxyacetone. The Edinburgh workers have found, as we have, that in the diabetic, where very little drop in the inorganic phosphate occurred after glucose, a marked fall followed the administration of dihydroxyacetone, and they state that it is difficult to reconcile these findings with those of Campbell, Fletcher, Hepburn, and Markowitz. Their respiratory metabolism experiments also show differences between dihydroxyacetone and glucose, and, as with our own data, these results are difficult to explain, if, as stated by the Toronto workers, dihydroxyacetone must be converted into glucose prior to its utilization. The Edinburgh workers conclude that dihydroxyacetone can be readily utilized by the diabetic.

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FAT METABOLISM IN DIABETES.*

I. THE BLOOD LIPIDS IN EXPERIMENTAL DIABETES.

By W. R. BLOOR, ETHELYN M. GILLETTE, AND MILDRED S. JAMES.

(*From the Department of Biochemistry, the University of Rochester School of Medicine and Dentistry, Rochester, New York.*)

Diabetes is regarded as primarily an abnormality of carbohydrate metabolism and the failure to utilize carbohydrate is the outstanding feature of the disease. Disturbance in protein metabolism is frequently present and is referred to a presumably normal process of transformation of about two-thirds of the protein molecule to carbohydrate with resulting wastage. Part of the remaining portion of the protein molecule may also be lost to the organism through a transformation into the acetone bodies. Disturbance in the fat metabolism is also frequently found in the severer forms of the disease, becoming evident in two ways,—by the appearance of the acetone bodies in the urine, regarded as mainly unburned normal residues of the fatty acids, and by the tendency to accumulate in the blood of fat and other derivatives of the fatty acids, such as phospholipids and also cholesterol. In well managed cases of diabetes treated by modern methods the increased percentage of fatty substances is frequently not great in even the severe cases, but the tendency toward abnormally high values may be noted in even the moderate ones, and many instances are reported in the literature of very high blood fat percentages, sometimes of 20 per cent or over. It is not evident that the accumulation of fat in the blood is disadvantageous but it is admitted by all that the dreaded coma is the result of the accumulation in the organism of unburned fatty residues.

In spite of these difficulties with fat metabolism and because of

the fact that fats are of all the food substances the best utilized by the diabetic, it has become the custom to allow increasingly large percentages of fat in the diet, and in some clinics, both here and abroad, such large amounts that any balance between the ketogenic and antiketogenic factors in the sense of Shaffer and Woodyatt was impossible.   Whether the large amounts of fat so fed were burned (1) or whether they were even completely absorbed is not beyond question.   Nevertheless clinically these high fat diets have been conspicuously successful over consider-able periods of time (2).

There are those, however, who believe that the last word has not been said regarding the large use of fat in diabetes, that although apparently successful, it may finally result in a break-down (3), a warning which has induced a feeling of caution in the minds of the more conservative clinicians.   In view of the importance of the problem a study of the abnormalities of fat metabolism in diabetes was called for, in order to find out if possible the mechanism of the disturbance and to determine the significance.   Experiments to elucidate these points were begun by Allen (3) and Wishart (4) some years ago and consisted in studies of the effect of fat feeding on the blood lipids of artificially diabetic dogs, but their data were lost as a result of the war. This type of research has seemed to us advantageous from several points of view.   The dog is accustomed racially to fat in the diet, eats it readily, and can digest large amounts of it, which disposes of the objection that the abnormality was produced by an unaccustomed diet; also since such large amounts can be taken, the strain on the mechanism would be more severe.   By removal of varying proportions of the pancreas any degree of diabetes can be produced and maintained.   One possible dis-advantage of the dog as an experimental diabetic is that acidosis is very difficult to produce and that death in coma from the condition is very rare.   Regarded in another way this fact is an advantage because it removes one more complicating factor and makes the interpretation of the findings easier.

The plan of the experiments was simple.   Normal young dogs with good neck veins were given a standard fat meal (ordinarily 100 cc. of olive oil in a thick emulsion with acacia) in place of their regular feeding, and blood samples were taken before the

meal and at 2 hour intervals thereafter for 8 hours. These samples were analyzed as described below. After two or three such experiments which thus served as controls, partial pancreatectomy was done[1] ordinarily with the removal of nine-tenths of the pancreas, leaving the portion nearest the ducts. After the wound had healed, similar feeding experiments were carried out on these animals during the remainder of their life. The diet, details of which are given in connection with the experiments, varied with the different animals but was always abundant. Little effort was made to prolong the life of the animals by dietary control or other treatment (although insulin was used in some cases), since the object in this first series of experiments was to observe the behavior of the blood lipids in untreated diabetes.

The methods for blood lipids used were the method of Bloor, Pelkan, and Allen (5) for total fatty acid and cholesterol, and the method of Bloor (6) for lipid phosphorus. Plasma alone was used. The nephelometer was one adapted from a Bausch and Lomb colorimeter according to a scheme previously described (6), but with the Bausch and Lomb solid glass cylinders with plane surfaces dipping just below the surface of the suspension in the tube so as to eliminate the meniscus.

Other data on the changes in blood fat after feeding were obtained by the method of Gage (7) whereby changes in the number of chylomicrons (blood dust, hemaconia) in the blood as a result of the fat feeding were obtained by use of the dark-field microscope. The chylomicrons are the fat particles suspended in the plasma, practically as they entered it from the thoracic duct. The chylomicron count followed in a general way the changes in the total fatty acids and free fat and served as a check on the chemical determinations. That fat is present in plasma in some other form than as chylomicrons was evidenced by the lack of parallelism between chylomicron count and free fat in some of the experiments, notably Experiment 3 below. The value "free fat" is a derived value and was obtained by subtracting the fatty acid combined in lecithin (two-thirds of the weight of lecithin) together with that combined as cholesterol esters

[1] We are greatly indebted to the following members of this school for these operations: Dr. J. R. Murlin, Dr. R. W. Angevine, and Dr. H. P. Smith.

(one-half of the weight of total cholesterol) from the value "total fatty acids." The value, "free fat" is subject to the error of all the determinations and is therefore an approximate value but it serves to indicate the changes in that constituent (the fat) which changes most in a fat feeding experiment.

The theoretical basis of the first of these methods, the nephelometric method for fatty acids, has recently been criticized by Blix (8). Most of the criticisms in his paper apply to the older method and are essentially the same as those made earlier by Csonka (9) which were taken into account in the modified method of Bloor, Pelkan, and Allen. However the latter part of the work, and particularly his Table II, is directed at the latter method and calls for comment. In this table Blix reports negative differences averaging 21 per cent (10 to 32 per cent) in comparing various mixtures of pure palmitic and oleic acids. Similar comparisons made with our apparatus and with reagents of undoubted purity gave values averaging about 12 per cent negative, confirming the essential correctness of Blix's results. In this connection however it should be noted that the fatty acids of blood are not simple mixtures of these two acids but something more complex, involving fatty acids regarding which we know relatively little, although palmitic acid and an oleic acid (whether ordinary oleic or not is unknown) are apparently present in large proportion (10). The blood fatty acid mixture has been found to have fairly constant melting point—slightly above the body temperature in all animals examined.

The standard of 60 oleic and 40 palmitic acids was adopted partly because it had a melting point near that of the blood fatty acids but mainly because, as was sufficiently shown (5), it gave results for fatty acids which compared with a good degree of accuracy with those obtained gravimetrically. As to that essential fact neither Blix nor Csonka makes any statement nor has it occurred to them to make any determinations to test it.

In the paper of Bloor, Pelkan, and Allen direct nephelometric comparison was not made between the standard and the fatty acids of the samples of blood used, it being thought sufficient that values by the nephelometric and gravimetric methods checked. However, in view of Blix's criticism, it was felt that something might be gained by such a comparison and it was made as below.

Normal dog plasma was digested on the steam bath (80°C.) for 12 hours with 20 per cent alkali. After cooling and acidifying, the digest was completely extracted warm with petroleum ether, the solvent washed with distilled water, and freed from unsaponifiable matter by the addition first of an equal amount of 93 per cent alcohol and then an equal amount of sufficiently alkaline water. Under these circumstances, the fatty acids remain in the watery alcohol as soaps, and the unsaponifiable substance is dissolved in the petroleum ether which separates at the top. After removal of the petroleum ether, the solution was washed once with more petroleum ether, and after removal of this was acidified and the fatty acids extracted with petroleum ether. After washing the solvent free from acid, it was removed by distillation and the fatty acids dried in a current of nitrogen. Samples of this fatty acid mixture were made up in alcohol so that 5 cc. contained 2.60, 2.03, 1.40 mg. of fatty acids. These were compared in the nephelometer with 5 cc. samples of the standard containing 2.00 mg. of a mixture of 60 parts of oleic acid and 40 parts of palmitic acid, and the results are given below.

Mixed Fatty Acids of Dog Blood.

Taken. mg.	Found. mg.	Deviation. per cent
2.60	2.59	−0
	2.63	+1.2
	2.55	−2.0
2.03	2.00	−1.5
	2.07	+2.0
	2.05	+1.0
1.40	1.45	+3.6
	1.41	+0
	1.47	+5.0

These results fall well within the limits originally claimed for the method and show that the 60 to 40 standard is very suitable for nephelometric measurement of the fatty acids of dog plasma. This applies, of course, to the fatty acid mixture present in dog blood in the postabsorptive state. Feeding olive oil as in the experiments below would introduce a considerable proportion of oleic acid, which gives nephelometric values somewhat lower than the acids of fasting plasma. The difference would not be

TABLE I.

Dog 24–81. Diet, Various.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylo-microns.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
		Experiment 1. Normal control. Weight 15 kilos.				
8.30	Clear.	3	85	302	254	104
10.20	Cloudy.	47	72	441	294	221
12.30	"	55	79	476	332	228
2.30	"	52	82	494	316	257
4.20	Milky.	99	69	595	374	322
		Experiment 2. 13 days postoperative. Weight 12.4 kilos.				
8.30	Clear.	4	148	640	410	317
10.35	Cloudy.	45	131	730	356	448
12.30	Milky.	109	160	910	330	637
2.30	"	65	175	580	284	332
4.20	"	73	188	740	406	407
		Experiment 3. 41 days postoperative. Weight 9 kilos. Urine sugar 16.2 gm. Blood sugar 0.433 per cent.				
8.45	Clear.	5	106	405	336	146
10.35	Cloudy.	15	103	510	312	268
12.35	Milky.	126	100	598	354	329
2.35	Very milky.	175	134	610	290	371
4.30	Milky.	107	119	563	266	345

History.—Dog operated upon May 20, 1925; nine-tenths depancreatized. Food after pancreatectomy May 20 to 30, meat and bread, producing marked diabetes with 18 gm. of urine sugar daily. Changed to high fat diet (carbohydrate 15, protein 10, fat 75 per cent) which quickly made the animal almost sugar-free. After 3 days on this diet (urine sugar 3.7 gm. daily), a standard fat feeding gave an essentially normal lipid reaction, although the lipid *levels* were much higher than in the control (Experiment 1). The animal was then (June 5) put on a mixed diet of carbohydrate 35, protein 27, and fat 38 per cent, and continued on this diet till the end, with resulting severe diabetes, high blood and urine sugar, and acetone body excretion. Death resulted (July 6) from a gas bacillus infection with abscess around the pancreas fragment. Experiment 3 on June 30, 41 days after operation, showed a somewhat increased reaction to the fat meal, the increase showing itself mainly in the free fat and chylomicrons.

TABLE II.
Dog 24-7. Diet, Protein.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
Experiment 1.	Normal control. Weight 14.3 kilos.		150 cc. olive oil.			
8.35	Clear.	14	119	500	478	142
10.30	Milky.	102	151	533	536	125
12.30	"	176	159	800	600	347
2.30	Very milky.	364	145	865	478	499
4.30	" "	215	156	· 605	486	229
Experiment 2.	Normal control. 150 cc. olive oil.					
8.35		6	113	448	348	178
10.30	Slightly milky.	55	122	508	366	223
12.30	Milky.	270	152	· 930	480	559
2.30	"	205	119	483	298	245
4.25	"	77	92	463	372	279
Experiment 3. 33 days postoperative. Weight 12 kilos. Urine sugar 44 gm. per day.						
8.40	Clear.	3	175	565	588	115
10.40	Milky.	85	171	1190	430	847
12.40	Very milky.	259	288	1530	468	1122
2 30	" "	270	260	1430	448	1045
4.25	" "	296	159	985	480	612
Experiment 4. 60 days postoperative. Weight 10.6 kilos. Urine sugar 22 gm. Blood sugar 0.22 per cent.						
8.45	Slightly cloudy.	24	75	585	438	268
10.45	Milky.	252	148	860	338	585
12.45	Densely milky.	541	113	1430	428	1106
2.45	Very milky.	347	189	1335	360	1032
4.45	Milky.	207	156	975	370	677
Experiment 5. 57 days postoperative. Effect of fasting 1 day.						
8.45	Milky.	231	105	1060	450	725
10.45	"	132	149	1040	466	680
12.45	Slightly milky.	52	106	465	382	176
2.40	Clear.	16	139	443	476	147
4.35	"	5	112	400	348	131

History.—Dog operated upon (nine-tenths depancreatized) May 24, 1924. After operation fed a high protein diet, largely lean meat and liver, with bone ash, on which the animal became strongly diabetic in about 30 days. The first fat feeding (urine sugar 9 gm.) on June 13 produced an essentially normal reaction of the blood lipids and so is not given in the table. The high protein diet (about 50 calories per kilo), was continued throughout, the animal losing weight but remaining in good spirits until just before the last experiment (July 22) after which it became more and more sluggish until sacrificed. The next day after Experiment 4 the urine sugar was down to 1 gm.

TABLE III.
Dog 25–60.   Diet, High Fat.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylo-microns.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
	Experiment 1. Normal control. Weight 21 kilos.					
7.50	Clear.	15	117	355	398	51
9.50	Very slightly cloudy.	38	123	340	402	93
11.50	Cloudy.	87	136	353	322	93
2.30	"	48	138	428	374	122
4.05	More cloudy.	29	135	425	344	151
	Experiment 2. 34 days postoperative. Weight 18 kilos. Urine sugar 13.8 gm. Blood sugar 0.270 per cent. Lipemia.					
8.30	Milky.	247	102	1055	280	834
11.00	"	141	157	710	400	391
1.00	"	267	132	782	474	422
3.00	Very milky.	262	159	937	426	600
5.00	Milky.	128	142	587	302	338
	Experiment 3. 41 days postoperative. Weight 17 kilos. Urine sugar 9.4 gm. Blood sugar 0.248 per cent.					
7.50	Clear.	8	111	617	414	304
9.50	Cloudy.	29	115	640	426	317
11.40	Milky.	420	148	1177	402	859
2.00	Very milky.	449	182		330	
4.00	" "	528	170	1613	330	1336
	Experiment 4. 46 days postoperative. Weight 16.8 kilos.					
8.10	Cloudy.	77	151	750	424	416
10.15	Almost milky.	126	145	742	256	523
11.50	Milky.	200	136	1027	304	779
1.50	"	152	113	1035	366	753
4.10	"	185	139	955	342	680

History.—Dog nine-tenths depancreatized, May 18, 1926. Food up to June 16 mixed diet, afterward and throughout the experiment, high fat (protein 40, carbohydrates 160, fat (suet) 720 calories—80 per cent fat calories—55 calories per kilo per day).   At the time of Experiment 2, after 4 days on the high fat diet, the animal was lipemic on the morning before the fat feeding.   During the next week on the high fat diet the animal showed no lipemia, but on June 28 a fat feeding gave an enormous reaction (Experiment 3).   Another experiment a week later (Experiment 4, July 2) gave a practically normal reaction.   On July 12 the animal appeared sick (blood sugar 0.24 per cent) and refused to eat.   Signs of distemper began to appear and to save the dog it was given insulin (10 clinical units per day) with, at first, marked improvement in condition, but the distemper returned and the dog died of it.

TABLE IV.

Dog 24–101. Diet, Various.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
	Experiment 1. Normal control. Weight 12.1 kilos.					
8.15	Clear.	11	112	308	290	77
10.15	Cloudy.	69	112	345	282	120
12.15	Milky.	84	113	390	402	84
2.20	"	33	128	425	428	84
4.15	Cloudy.	30	121	435	404	125

Experiment 2. 20 days postoperative. Weight 11 kilos. Blood sugar 0.167 per cent.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
8.45	Cloudy.	50	218	515	482	120
10.50		121	234	730	494	322
12.45	Very milky.	165	219	943	456	466
2.50	Milky.	99	189	550	472	173
4.55	Cloudy.	81	183	495	474	118

Experiment 3. 31 days postoperative. Plasma lipemic. Blood sugar 0.262 per cent. Urine sugar 9 gm.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
9.15	Milky.	113	236	1105	632	604
11.20	"	190	189	1110	600	647
1.30	Thick with fat.	231	223	1475	644	971
3.30	" " "	248	174	1275	812	600

Experiment 4. 42 days postoperative. Weight 10.0 kilo. Blood sugar 0.233 per cent. Urine sugar 12 gm.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
8.20	Milky.	146	257	1210	512	771
10.30	Like cream.	388	297	2525	872	1844
12.45	Thick "	375	262	2500	1072	1699
2.25	Opaque.	500	298	2325	900	1724
4.15	White with fat.	600	341	3288	856	2604

Experiment 5. 64 days postoperative. Weight 9 kilos. Blood sugar 0.301 per cent. Acetone bodies in urine.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
8.25	Clear.	10	158	548	322	281
10.15		4	149	513	292	265
12.05		11	141	573	486	202
2.15		7	171	618	452	259
4.15	Slightly cloudy.	19	143		448	242

TABLE IV—*Concluded.*

History.—Dog nine-tenths depancreatized, September 29, 1925.   Given 4 to 5 clinical units of insulin daily until October 12 to aid healing of wound, and none thereafter.   Diet, during recovery, mixed animal house diet. From October 9 to 19, high fat diet as follows: bread 100, liver 50, suet 700 calories—65 calories per kilo—75 per cent fat.   Experiment 2 on October 19, after 9 days on the high fat diet.   Beginning October 24, the diet was changed to bread 250 ,liver 70, suet 360 calories (62 calories per kilo—53 per cent fat) in order to increase the severity of the diabetes.   After 6 days on this diet, Experiment 3 was carried out.   At this time, the animal was lipemic (1.1 per cent of total fatty acid) and markedly diabetic, with blood sugar of 0.262 per cent and 9 gm. daily of urine sugar.   The feeding of the standard dose of fat produced a greater lipemia.   This diet was continued till the next experiment 11 days later (Experiment 4).   At this time, the plasma of the animal was quite milky (1.2 per cent fat) and the fat feeding produced a further increase—a very marked lipid reaction.   From this time on, the animal was on a carbohydrate-protein diet, becoming more and more emaciated and ill.   On December 2 and again on December 16, fat feeding experiments were made, but the reactions were slight, possibly because of failure to digest and absorb, because, at this time, the feces contained noticeably large amounts of fat.   The animal was sacrificed when obviously moribund.

TABLE V.

Dog 24–6.   Diet, Protein-Carbohydrate.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylomicrons.	Cholesterol.	Fatty acids.	Lecithin.	Free fat.
Experiment 1. Normal control. Weight 14.2 kilos. Fed 150 cc. olive oil.						
8.30	Clear.	10	111	413	608	0
10.30	Milky.	150	126	545	812	159
12.30	Very milky.	146	79	698	362	437
2.30	Milky.	87	85	580	439	260
4.30	"	154	171	630	463	265
Experiment 2. 31 days postoperative. Weight 10.1 kilos. Urine sugar 44 gm. per day.						
8.45	Cloudy.	11	88	510	296	283
10.40	Milky.	122	120	683	410	369
12.40	Very milky.	354	107	1580	506	1206
2.35	" "	286	174	1440	478	1074
4.30	Milky.	183	199	1505	468	1127

TABLE V—*Concluded.*

Time.	Plasma.	Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.

Experiment 3. 40 days postoperative. Weight 9 kilos. Urine sugar 40· gm. per day. Blood sugar 0.390 per cent.

Time.	Plasma.	Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
8.45	Milky.	6	155	888	292	642
10.30	Slightly milky.	131	229	425	410	75·
12.40	Milky.	265	145	1105	456	905
2.35	Very milky.	327	186	˙ 1585	560	1149
4.30	Opaque.	330	.137	2250	438	1912

Experiment 4. 46 days postoperative. Lipemia (total fatty acids 3 per cent). Blood sugar 0.273 per cent. Urine sugar 34 gm. Weight 8.3 kilos.

Time.	Plasma.			Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
9.10	Opaque with fat.			672	178	3025	526	2616
11.05	White	."	"	1151	473	3925	620	3353
1.00	"	"	"	1289	284	3734	596	3247
3.00	Dense	"	"	1287	298	4267	428	3895
5.00	"	"	"	1365	183	3075	490	2688

History.—Dog operated upon May 24, 1924; nine-tenths depancreatized. Weight before operation 31 pounds, 3 ounces. Diet after operation, cooked lean meat or liver and dog biscuit. Throughout the experiment on this diet the animal lost weight rapidly until at 24 days after operation its weight was 24 pounds, 3 ounces. At this time the urine volume was 1100 cc. with urine sugar of 15 to 20 gm. per day. A fat feeding experiment on the 24th day after the operation gave an essentially normal reaction and is not reported. A week later, however, a markedly abnormal reaction was obtained (Experiment 2, June 24). At this time the urine sugar was 44 gm. per day, the dog thin (22 pounds, 8 ounces) but lively. By July 3, the time of Experiment 3, the animal's weight was 19 pounds, 12 ounces and it was becoming weak. The urine sugar was 40 to 60 gm. per day and on the day of the experiment the blood sugar was 0.390 per cent. After Experiment 3 the protein-carbohydrate diet was continued, the animal becoming weaker up to the time of the final experiment on July 9. At this time the animal was very lipemic (total fatty acids of plasma 3 per cent), had a blood sugar of 0.273 per cent, a urine sugar of 34 gm., and weighed 18 pounds, 5 ounces, a loss in weight since operation of about 13 pounds. Next day the dog was moribund and was killed under ether.

TABLE VI.

Dog 25–10.   Diet, Various with Insulin.

Mg. per 100 cc. of plasma.

Time.	Plasma.	Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
Experiment 1. Normal control. Weight 14.2 kilos.						
8.30		2	81	320	366	49
10.35		100	100	378	362	103
12.35		40	101	375	466	31
2.30		74	106	372	458	31
4.30		50	112	326	472	25

Experiment 2.   90 days postoperative.   Weight 8.7 kilos.   Blood sugar 0.277 per cent.   Urine sugar 19 gm.

Time.	Plasma.	Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
8.40	Clear.	30	143	480	392	99
10.40	Cloudy.	47	82	575	346	317
12.30	Milky.	191	149	1048	356	761
2.30	Very milky.	315	147	1032	310	787
4.30	Cloudy.	112	152	565	390	254

Experiment 3.   130 days postoperative.   Weight 9.4 kilos.   Blood sugar 0.252 per cent.   Urine sugar 5 gm.

Time.	Plasma.	Chylomi-crons.	Choles-terol.	Fatty acids.	Lecithin.	Free fat.
8.00	Cloudy.	10	264	905	336	594
10.00	"	7	234	756	344	449
12.00	Very milky.	211	257	1312	464	923
2.00	Creamy.	411	257	4511	426	4142
4.00	Very fatty.	377	368	5300	404	5163
6.00	" "	454	469	5660	506	5167

History.—Initial weight 14.2 kilos. Operation, October 29, 1925; eight-ninths depancreatized. Insulin, 4 to 6 rabbit units daily given until the wound had healed (November 13). Diet, bread and liver once a day (60 calories per kilo). Probably because of the larger portion of pancreas left in place, the diabetic condition developed very slowly, so that 37 days after the operation the blood sugar was still normal and fat feeding experiments gave essentially normal reactions in the blood lipids. By the 45th day, however, diabetes was well developed with a blood sugar of 0.254 per cent and a daily sugar excretion of over 20 gm. The weight had dropped to 11.1 kilos, but a fat feeding experiment at this time gave an essentially normal reaction. By the 67th day the blood sugar had reached 0.313 per cent and the animal appeared emaciated. A fat feeding experiment gave again a normal reaction. The diet was then changed to fat 80, carbohydrates 15, and protein

great and a correction, if applied, would make still more striking the differences found.   ‚

Blood sugar and urine sugar were determined by Benedict's methods (11, 12).

In all eleven animals operated upon, of which three died as the result of the operation from various causes, two could not be made diabetic by overfeeding with carbohydrate, leaving six animals on which long time experiments were made. All of these showed definite abnormalities in the blood lipids, and in five of them these abnormalities were very marked. Since it would hardly be possible, even if desirable, to give the results of all the experimental data, only those experiments which showed the abnormalities most strikingly were given, and, of the control experiments on the animals when normal, only the ones which showed the greatest reaction to the fat feeding. The data on

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5 per cent of the caloric intake (80 calories per kilo), and after 7 days on this diet the standard fat feeding gave again an essentially normal reaction. The animal was emaciated and ravenous and the same diet was raised to 100 calories per kilo. After 8 days on this diet, on the 90th day after pancreatectomy, the first definitely abnormal plasma lipid reaction to the standard fat dose was obtained (Experiment 2). The animal at this time was very thin (weight 19 pounds—loss of 12 pounds from initial weight) with blood sugar of 0.277 per cent and a daily urine sugar of 18 gm. Because of the danger of losing the animal, insulin was given, 4 to 5 rabbit units daily, with resulting marked improvement in appearance and spirits. The high fat diet was continued but it was noticed that much fat appeared in the feces. An experiment on February 4 with blood sugar 0.200 per cent gave a normal reaction. On February 16 the diet was changed to carbohydrate 33, protein 33 and 125 calories per kilo, and the insulin increased to 12 units daily. On March 7, the animal had gained in weight 900 gm. and was bright and lively. Insulin was omitted on this day and on March 8 when another fat feeding experiment, No. 3 (2 days without insulin), was made. Blood sugar 0.253 per cent. The blood lipid reaction to this feeding was very great, the total fatty acids reaching 5.66 per cent and the cholesterol showing a marked increase. After this experiment the animal was continued on a protein-carbohydrate diet with insulin with three further interruptions for fat feeding experiments, only one of which, on March 31, gave a defi-' nitely abnormal reaction, apparently about enough insulin being given to keep the fat metabolism approximately normal. In this one, the total fatty acids increased from 0.500 to 1.2 per cent at the 8th hour. Cholesterol and lecithin were little affected. Before further experiments could be made the animal died of what was apparently insulin shock, from which it could not be rescued by intravenous injection of dextrose.

the fat feeding experiments are collected in Tables I to VI which are arranged in the order of the greatness of the abnormality. Unless otherwise noted, the test meal was always 100 cc. of olive oil in a thick acacia emulsion flavored with meat extract, given immediately after taking the first blood sample.

Analysis of the Experiments.

Two things were kept in mind in considering the data—the changes in the fasting levels of the various lipids of the plasma as the disease proceeded and the changes in the amounts of the lipids as the result of the single fat feeding—the reaction to the fat dose.

Table I, Dog 24-81.—This animal died of an infection before any great abnormalities manifested themselves. There is to be noted the increased (60 to 110 per cent) fasting level of all the blood lipids on the high fat diet, the level falling again on the mixed diet although not to the normal. The effect of the standard fat meal on the blood lipids was not definitely abnormal at any time although the animal was severely diabetic with a blood sugar of 0.433 per cent.

Table II, Dog 24-7 (Protein Diet).—This animal was kept on a high protein diet throughout its life after the operation. Two normal control experiments are reported because they were given on successive days with $1\frac{1}{2}$ times the standard dose of fat. The results were essentially the same in both, showing that the fat feeding on one day had no effect on the feeding the next day. The two fat feeding experiments on the animal when diabetic showed a markedly abnormal effect of the fat dose on the plasma fat (glycerides) as shown by the chylomicron count, the total fatty acids, and the free fat, both as regards fasting level and increases in the 8 hour period after fat feeding. Cholesterol and lecithin were little affected. Thus, the total fatty acid fasting level was 500 and 448 mg. per 100 cc. of plasma in the animal when normal, and 565, 585, and 1060 mg. in the diabetic animal, the last plasma being lipemic. The free fat was 142 and 178 mg. in the normal and 115, 268, and 725 mg. in the animal when diabetic. Cholesterol was 105 mg. per 100 cc. in the lipemia sample and 119 and 113 mg. in the normal. Lecithin was 450 mg. as compared with 478 in the normal although both lecithin

and cholesterol had reached higher values than those in Experiment 3. The maximum increase resulting from the fat dose on the total fatty acids and free fat in the normal animal had been 482 mg. (110 per cent) and 357 mg. (250 per cent) respectively, while in the diabetic state the maximum increases were 965 mg. (170 per cent) and 1007 mg. (975 per cent) respectively. Some interesting details came out of this experiment. For instance, after a fat feeding on June 14, when the urine sugar was 25 gm., the next day the urine sugar was down to 1 gm. A fast day, when the animal was lipemic (Experiment 5) produced a great fall in the blood lipids (except cholesterol), resulting in a clear plasma at the end of the day. The urine sugar had fallen from 44 gm. to 19 gm. the next day.

Table III, Dog 25–60 (High Fat Diet).—This animal was on a high fat diet (80 per cent of the calorie value of the diet as fat) throughout the experiment after recovery from the operation. The fasting levels of all the blood lipids (excepting lecithin which did not change markedly), were higher in the diabetic state than in the normal—total fatty acids reaching 75 per cent above, cholesterol 33 per cent, and free fat 1500 per cent above the level in the animal when normal. The reaction of the blood lipids in the diabetic state to the standard fat dose was also markedly greater than in the animal when normal. Thus, the increase of the total fatty acids above the fasting level in the normal was 21 mg. (20 per cent), while in the diabetic state an increase of 996 mg. (160 per cent) was noted in Experiment 3. The greatest rise in cholesterol noted was 64 per cent in Experiment 3 as compared with 20 per cent in the control. In the case of lecithin, the fat feeding generally produced a fall of plasma lecithin except in Experiment 2, where the lecithin level was low to begin with. In Experiment 2, where the animal was lipemic to start with, the fat feeding produced at first a *fall* in the total fatty acids, free fat, and chylomicrons, while the lecithin and cholesterol rose. Later the total fatty acid values came back to practically the initial level. This fall may possibly be explained by the inflow of fat from the intestine stimulating to activity a sluggish outflow mechanism, resulting in an outflow for a time faster than the inflow.

The diabetic state in this animal resulted in a higher fasting

level of all the blood lipids, most noticeable in the total fatty acids and free fat and less in the cholesterol and lecithin which were not higher than the normal until 46 days after the operation. The fat feeding produced an effect on the blood lipids much greater than in the normal, a value of 1.6 per cent for total fatty acids being reached in Experiment 3.

Table IV, Dog 24–101 (Various Diets).—After 9 days on the high fat diet which, according to Blix's (2) results with man should have adapted the dog to the diet, the feeding experiment (Experiment 2) showed a higher fat level in the blood to begin with (all lipids) and a more marked rise in total fatty acids and free fat than in the animal when normal.   Thus, total fatty acids increased 328 mg. (60 per cent) as compared with 127 mg. (40 per cent) in the normal.   Cholesterol and lecithin changed but little.   Increasing the protein and carbohydrate and cutting down the fat in the diet increased the severity of the diabetes and when, after 6 days on this diet, Experiment 3 was carried out, the animal was lipemic with total fatty acid fasting level $3\frac{2}{3}$ times the normal value and lecithin and cholesterol levels over twice the normal. A fat feeding at this time resulted in increases of total fatty acids of 370 mg. as compared with 127 mg. when normal.   Cholesterol diminished and lecithin increased.   On the same diet, 11 days later, Experiment 4 showed an enormous reaction to the fat dose, the total fatty acids increasing 2000 mg., the lecithin doubling, and the cholesterol increasing about 70 per cent.   From this time on, various diets were experimented with, the animal continuously losing ground.   Fat feeding experiments during this period produced little effect on the blood lipids (Experiment 5), perhaps because the animal could no longer digest the fat.

Table V, Dog 24–6 (Protein-Carbohydrate Diet).—This series of experiments gave an example of the behavior of the plasma lipids in severe diabetes on an uncontrolled protein-carbohydrate diet. Disturbance of fat metabolism, as shown by the plasma lipids, did not take place until the animal was severely diabetic with hunger, rapid loss of weight, marked glycosuria, large urine volume, and blood sugar above 0.300 per cent.   The disturbance showed itself first in an abnormally great reaction to the standard fat meal (Experiment 2), then in continuous lipemia and still more marked reactions to the fat meal (Experiments 3 and 4).

The most marked effect was on the total fatty acids and free fat of which the changes in fasting level and the excursions in values were very great   Thus, in Experiment 4, the fasting level for total fatty acids was 2600 mg. above the normal fasting level and the increase in total fatty acids after the standard fat meal was 1240 mg. as compared with 280 mg. increase in the control. These changes were reflected in the free fat and in the chylomicron count.   The cholesterol fasting level was raised toward the end of the experiment.   The lecithin fasting level was high only in the last experiment.   The excursions in values of lecithin and cholesterol after the fat dose were noticeably greater than in the normal controls only in the last experiments.   The fall in total fatty acids at the 2nd hour in Experiment 3 may be the result of an analytical error, but, since in control feeding experiments on this animal when normal, similar drops in values were encountered, and since another analysis of this extract gave similar figures, the value is believed to be correct.   This fall as the result of feeding may probably be explained on the same basis as a similar fall in Experiment 2, Table III, as the result of the stimulation to activity of the outflow mechanism as the result of the inflow of fat from the intestine.   The fact that both lecithin and cholesterol had risen markedly in the same period may be significant.

The series of experiments on this animal exhibits very well both the increase in fasting level of the fat and the increased reaction to the fat meal which appears to be characteristic of the disturbed fat metabolism in the diabetic state.   Also what appears to be equally characteristic is that changes in the cholesterol and lecithin levels and responses to the fat dose are irregular and may be missing altogether.

Table VI, Dog 25–10 (Mixed Diet).—This animal was notable in that with a somewhat larger pancreas fragment (one-ninth instead of one-tenth as in the others), the diabetes was very slow in developing.

12 days after the operation, a 3 day feeding of a high fat diet (fat 80, carbohydrate 15, protein 5 per cent) before a fat feeding experiment (not reported in table) resulted in a raising of the *fasting level* of the plasma fatty acids and cholesterol (total fatty acids from 413 to 545, cholesterol from 111 to 216 mg. per 100

cc.), but the increase of the blood lipids after the standard feeding was within normal limits.   This animal did not show any marked abnormality in the blood lipid level or in the reaction to the standard dose of fat, even when very diabetic, with a blood sugar of 0.313 per cent.   At this time, the animal was evidently about to die and to save it insulin was given, resulting in great improvement with gain of weight.   While under insulin, no abnormal lipid reaction was obtained.   As the result of withdrawing insulin for a 2 day period on a diet of equal caloric amounts of protein, carbohydrate, and fat, the standard fat dose gave an enormous reaction, chiefly in the fat (glycerides)—total fatty acids 4700 mg. increase from a fasting level which was about 3 times the fasting level of the animal when normal, cholesterol increase of 205 mg. per 100 cc. from a fasting level also about 3 times the value for the animal when normal.   Lecithin was also increased but only about 50 per cent above a fasting level, which was practically normal.   Later experimentation under insulin gave reactions to the standard dose of fat which while abnormal were not greatly so and are not reported.   The outstanding features of work with this animal were the slow development of the diabetes, the fact that it would probably have died of the disease without showing marked abnormality of fat metabolism, and the fact that after being restored with insulin a short deprivation resulted in the enormous lipid reaction noted.

Summing up the results on all the animals, the experiments indicate that the changes in the blood lipids due to the artificially produced diabetic state were as follows: (1) An elevation in the postabsorptive level of the blood lipids eventually reaching the stage of visible milkiness (lipemia).   The constituent most consistently elevated was the fat (glycerides) with cholesterol next and lecithin least.   (2) A heightened response of the blood lipids to the standard fat meal, again noticeable mainly in the fat with cholesterol following and lecithin least affected.

The extreme disturbances—lipemia and greatly heightened reaction of the blood lipids to the standard fat dose—were followed in most of the animals by a period of sickness during which fat absorption did not take place well and in which there was consequently no abnormal response to the fat dose and which was in turn generally followed by death.   Since these results were

obtained in all the animals but one (which died of an infection early) and on a considerable variety of diets including a high fat diet and diets (protein and protein-carobohydrate) in which fat was present only in small amounts, the disturbances seem to be independent of diet. The abnormalities occurred only when the animals were very diabetic with high blood sugar and much wastage of sugar in the urine. On the other hand, high blood and urine sugar did not necessarily mean great increases in blood lipids as, for example, in the dog in Table I, Experiment 3, in which the blood sugar was 0.433 per cent without any more striking abnormality than a somewhat increased blood lipid level, and in Table V in which the animal had a blood sugar of over 0.25 per cent (not recorded) for more than 30 days without any marked abnormality in the behavior of the blood lipids.

DISCUSSION.

The literature on the topic of fat metabolism in diabetes is very large and a summary of it will not be attempted in this paper. Three pieces of work of relatively recent date will, however, be especially considered in the discussion of the present report and in these will be found a full discussion of the literature. These are the careful work and analyses of Gray (13) on the blood lipids of human diabetes, the results of which may be summed up as follows: Strikingly high blood fats were astonishingly infrequent, 90 per cent having blood fats below 1.5 gm. per 100 cc. (total fat by Bloor's original method), but the blood fat was above normal in diabetes with a consistency equal to the blood sugar, 78 per cent having blood fats above the level of high normals, while 72 per cent had blood sugar above the high normal for blood sugar. The longer the duration of the disease before examination the lower the blood fat, presumably because only those patients live long who have low fats. The higher the blood fat level the shorter was the expectancy of life. The method made use of by Gray was the original total fat determination of Bloor which included both total fatty acid and cholesterol in the nephelometric measurement. Cholesterol has since been shown (9) to give considerably higher results than the fatty acids in the nephelometer and therefore the values reported would be higher than they ought to be. However, the comparison was made with

values from normal individuals similarly determined and since cholesterol lags behind the fatty acids in the increase of values found in diabetic blood, the difference between normal and diabetic blood would be if anything more marked than shown by Gray.

The equally extensive and careful work by Blix (2) using a different fat method with patients on a high fat diet, some of them over long periods of time, led him to the following conclusions: In severe diabetes, a high degree of hyperlipemia is a rare symptom, moderate or slight hyperlipemia a relatively frequent one. Hyperlipemia was found in all of six cases of coma but in extremely varying degrees. Hyperglycemia was in most cases a more sensitive symptom of the disturbed metabolism than the hyperlipemia. High fat diet had no relation to hyperlipemia, created no increased susceptibility to hyperlipemia, and often caused a lowering of a lipemia already present as was also reported by Marsh and Waller (14) and Blatherwick (15). A transient rise in the blood fat level was found both in normals and in diabetics during the early days of a high fat diet but later in both normals and diabetics an adaptation took place after which the high fat diet did not produce hyperlipemia (16). Susceptibility to hyperlipemia in severe diabetes varied widely with individuals. After the ingestion of excessive quantities of fat, a marked hyperlipemia may develop in the diabetic. With these exceptions diabetic hyperlipemia appeared to be quite independent of food fat. The hyperlipemia was secondary to the disturbance in carbohydrate metabolism. There was no close relation between the magnitude of the blood fat rise after fat absorption and the degree of active diabetic symptoms and in most instances it was not greater than in normals on the diabetic diet. A fat feeding might cause a fall in an established hyperlipemia. An abnormally slow rate of outflow does not explain diabetic hyperlipemia. Blix adds the following significant information. In one patient (Case 3) the hyperlipemia was slight when the blood sugar remained below 0.15 per cent but became pronounced when the blood sugar reached 0.20 per cent. In fat feeding to diabetics, the increase of blood fat was higher, with a blood sugar of 0.20 per cent, than when the blood sugar was below that value, but otherwise there was no definite relation between the fat absorption curves and

the activity of the symptoms. He found no relation between the magnitude of the absorptive rise and the initial blood fat level. The fall of blood fat after insulin may follow closely that of blood sugar, although certain individuals were found in whom there was a fixation of the fat metabolism and who did not respond to insulin by a lowering of the blood lipids.

It should be remarked that the method used by Blix (Bang's method with the p-fraction) shows only a portion of the blood lipids—that part soluble by contact of petroleum ether with the blood, air-dried on absorbent paper, which is stated to consist of free fat and free cholesterol. While his determinations give only a partial picture of the blood lipids and therefore cannot be closely compared with the data in the present paper, they probably include that part of the lipids which was most subject to change (free fat) and can be taken as representing fairly well the changes in level of the blood lipids under different conditions.

The third series of investigations, having a close bearing on the present work was that of Allen (3) on the effects of high fat feeding on experimentally diabetic dogs. His conclusions may be summed up as follows: Severely diabetic dogs, which digest high fat diets, regularly develop some degree of abnormal lipemia and, in a minority, this lipemia becomes extreme (15 per cent or more of blood fat). Apart from a sufficient supply of fat in the diet, the one indispensable prerequisite for diabetic lipemia is the existence of active severe symptoms in the form of glycosuria and hyperglycemia. Diabetic lipemia evidently represents some secondary breakdown in fat metabolism not directly connected with the endocrine function of the pancreas and not due merely to excess of fat in metabolism or to loss of sugar from the body. There are wide variations in individual susceptibility to this disorder among both animals and patients. One-sided fat diets produced fatal disturbances but did not produce any significant grade of lipemia in dogs not severely diabetic. In severely diabetic dogs pushing the fat too high resulted in loss of appetite or cachexia.

The results obtained in the present series of studies on experimental diabetes agree with most of the findings expressed in the statements above and in general with the reports on the blood lipids of diabetes in the literature. The most characteristic

feature is always the elevation of the postabsorptive level of the blood lipids and of these the fat (glycerides) is the constituent most markedly affected.   In human diabetes the cholesterol level is characteristically high; in fact, so regularly so that it has been suggested as an indicator of the blood lipid level.   In our series, the cholesterol level was often not abnormally high over considerable periods of the diabetic life of the animals.   Since these animals were on a relatively low cholesterol diet, a dietary factor in the cholesterolemia of humans may be suspected.   As is the case in human diabetes, the lecithin level is least affected by the diabetic state.   Of the other abnormalities in the blood lipids—the increased reaction to a fat meal and the lipemia, the first has been but little studied in human diabetes although it is undoubtedly present (17).   Blix (2) did not find it in his series of treated cases if they had been allowed time to become habituated to the high fat diet.   Lipemia was a frequent phenomenon under the older systems of treatment of diabetes and, in such of these as have been studied, the relative proportions of the blood lipids are of the order of those reported in this paper.   These extreme abnormalities cannot be said to be characteristic of the diabetic state, since most of the animals were severely diabetic over long periods without showing either.   On the other hand they were found in all but one of the animals at some stage of the disease and it is significant that these extreme states were followed by a period in which the animals were obviously very ill and which was in turn soon followed by death.   The prognosis in similar states in human diabetes has always been bad (13, 18).

As to the reason for the lipemia or the origin of the fat producing it, little can be said.   Since lipemia occurs on a low fat as well as on a high fat diet, it cannot be ascribed entirely to the fat of the food, but it is well recognized that the fat of the stores may be readily drawn on by the blood.   As to the reason for the accumulation in the blood, the earlier explanation of a diminished rate of outflow is not adequate in view of the fact, reported several times in the literature and noted above, that feeding of fat may cause a lowering of the blood fat.   The facts point rather to a sluggish removal mechanism which requires a high concentration of fat in the blood to start it working but which is then fairly effective.   Consideration of the data recorded above and of the

facts in the clinical literature lead to the conclusions that a high fasting level of the blood lipids especially of the fat (glycerides) is characteristic of the severely diabetic state. The more striking manifestations of a disturbed fat metabolism, lipemia and increased response of the blood lipids to a fat meal, while found in five out of the six animals reported upon above and of frequent record in the older literature on human diabetes with generally fatal outcome, appear to be less characteristic and may possibly be avoided by suitable treatment. Work on this point and also having to do in more detail with the mechanism of the disturbances of fat metabolism in diabetes is in progress.

It is a pleasure to acknowledge the unfailing interest and advice throughout this work of Dr. E. P. Joslin of Boston.

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VITAMIN STUDIES.

XVI. VITAMIN A IN EVAPORATED MILKS MADE BY VACUUM AND AERATION METHODS.*

BY R. ADAMS DUTCHER AND HANNAH E. HONEYWELL,

(From the Department of Agricultural and Biological Chemistry, Pennsylvania State College, State College.)

AND CHESTER D. DAHLE.

(From the Department of Dairy Husbandry, Pennsylvania State College, State College.)

The first paper of this series (1) described the results obtained in an attempt to determine whether or not vitamin B is destroyed when cow's milk is subjected to commercial methods of evaporation.

The study described in the present paper, which is the second in the series, was undertaken with the view of answering a similar question with regard to vitamin A. We pointed out in the previous paper, to which we have just referred, that:

"In the past practically all of the data available, relative to the vitamin content of evaporated milks, have been obtained on samples the past history of which has not been known. Furthermore, few attempts have been made to compare the evaporated milks with the raw milks from which they were made. As a result few authoritative data have been obtained concerning the influence of commercial evaporation processes on the stability of the various vitamins.

The present studies were undertaken with the view of preparing evaporated milks under conditions approximating, as closely as possible, the conditions used in commercial practice and comparing these products with the original milk from which they were made, to eliminate the effect of any fluctuation in vitamin content that may take place in raw milk obtained from various sources and at different seasons of the year."

Evaporated Milks.

For details of the manufacturing processes which were carried out on a semicommercial scale in our college creamery, the reader is referred to our former paper (1).   For the sake of clearness, however, it is necessary to say that two types of evaporated milks were studied: (1) evaporated milk made by the vacuum process, and (2) evaporated milk made by the aeration process.   The former was representative of most commercial evaporated milks sold on the market, having been made in the copper vacuum pan in the absence of air.   The aerated milk was representative of a type which is of less commercial importance in the evaporated milk industry but was studied because of the scientific interest associated with vitamin destruction by oxidative processes. Portions of both of these types of milks were sterilized by the fractional sterilization method.   These sterilized samples were studied with the view of determining the effect of sterilization as it is practiced in all condenseries where evaporated milks are made.

The certified milk, from which the evaporated milks were made, was obtained fresh from the barn every morning.   The evaporated milks, which were made at 4 or 5 week intervals, were analyzed and diluted to the concentration of the original raw milk before they were fed.   In other words, all evaporated milks were compared directly with equal volumes of the certified milk from which they were made, the latter being used as the standard.

In most of the work reported by other workers it has not been possible to say with certainty whether or not vitamin A destruction occurred during commercial evaporation, owing to a lack of knowledge regarding the vitamin content of the original raw milk, which is known to fluctuate in vitamin potency depending on the vitamin content of the dairy ration (2).

As a result, five types of milk were fed: (1) raw milk from the certified herd (R. M.), (2) evaporated milk made by the vacuum process (V. E.), (3) evaporated milk made by the vacuum process and sterilized (V. E. S.), (4) evaporated milk made by the aeration process (A. E.), and (5) evaporated milk made by the aeration process and sterilized (A. E. S.).

During the year 1925 a study was made using the old prophylactic method of feeding.   The rats, which numbered 150, were fed separately in individual cages and on screens.   The rats were

weighed weekly and food intake records were kept for each individual.

The ration, which was not irradiated, consisted, in parts per 100, of casein 18, agar 2, McCollum's salt mixture[1] (No. 185) 3, and dextrin 77.   To insure an adequate and constant supply of vitamin B, 0.5 gm. of Fleischmann's special dried yeast was fed (daily) separate from the ration.   The experimental milks were fed from the beginning of the experiment in special feeding dishes and no difficulty was experienced in obtaining complete consump-
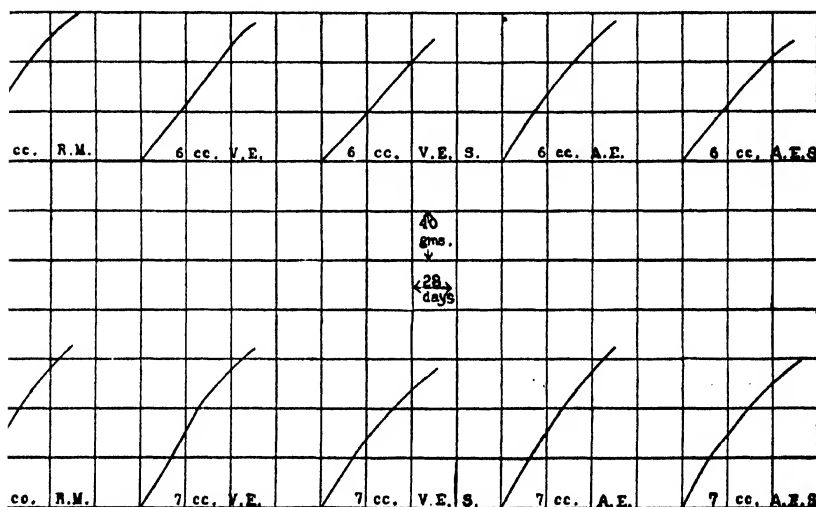
CHART 1. Vitamin A in raw and evaporated milks as measured by the prophylactic method of feeding.

tion of the milk.   No attempt was made at that time to provide for a supply of the antirachitic factor.   Chart 1 summarizes the results obtained by the prophylactic method.   Each curve represents the averages of ten or more animals and the abbreviations refer to the different types of experimental milks which were described in a previous paragraph.

Previous work, in which the prophylactic method of feeding (2) was used, showed that approximately 10 cc. of raw milk are necessary to furnish sufficient vitamin A for normal growth in

[1] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

rats.   Later work, in which the prophylactic method was used, but with the ration irradiated, gave us results which showed that considerably less milk was required for fairly satisfactory growth. This is in agreement with the work of Chick and Roscoe (3) who noted that a smaller amount of milk was required to furnish sufficient vitamin A when the curative method was used and when the ration contained irradiated vegetable oil.   These workers, using the normal rate of growth as their standard, endeavored to find the amount of milk necessary to produce an average gain of 10 to 13 gm. per week for a curative period of 4 weeks following the prefeeding period on the vitamin A-free diet.

Sherman and Munsell (4) have used similar technique, with the exception that they have felt that it is not necessary to take special precautions regarding the furnishing of the antirachitic factor by irradiation.   The Sherman-Munsell standard also differs from that of Chick and Roscoe in that Sherman and Munsell make no attempt to obtain the normal growth rate during the curative period.   Instead, they have sought to find the minimum quantity of food which will furnish sufficient vitamin A to prevent loss of body weight and produce a slight but steady growth.   They have suggested that this technique be standardized by finding that amount of food which will produce an average weekly increase in body weight of 3 gm. for a period of 8 weeks following the depletion of the body reserves.

Javillier and coworkers (5) have suggested a similar but higher standard.   Using the curative method also, they attempt to feed that amount of food which will produce, for 30 days, an increase in body weight of about 30 per cent and an angle of growth of 30 degrees when days and gm. are given the same linear value on the chart.   This standard is what might be termed, equivalent to about one-half of normal growth.

EXPERIMENTAL.

In 1926 we repeated our experiments but modified the technique by using the curative method of feeding and the basal ration was irradiated in shallow pans with a Cooper Hewitt Uviarc lamp at a distance of 24 inches for 15 minutes, the ration being stirred at intervals of 5 minutes during the irradiation.   Previous work in our laboratory (6) has shown that dextrin prepared from commercial corn-starch can be activated to furnish sufficient anti-

rachitic factor to eliminate the possibility of insufficient calcium and phosphorus utilization. We have not yet made a study of this phase of the problem but we feel that the activation of our dextrin is probably due to the presence of sterols in the impure commercial starch from which the dextrin was made.

A total of 177 rats was used in this portion of the work, all animals being fed on screens and in individual cages. The basal ration was of the same composition as that described in the discussion of our 1925 experiments and Fleischmann's[2] special yeast was fed daily in individual dishes separate from the ration.

All rats were placed on experiment at 21 days of age at a weight of about 40 gm. Animals which weighed less than 37 gm. or more than 42 gm. were not used. These precautions were taken with the hope that we might obtain more uniform responses to our experimental procedure. Our stock ration (7) is of constant composition and we should expect that vitamin storage would not vary appreciably in our experimental rats.

This expectation has been borne out by our experience during the past year. A large proportion of our rats, when placed on the vitamin A-free basal ration will have depleted their vitamin A body reserves in 35 to 40 days. By far the greatest number will have reached this point on the 37th day of the prefeeding period, at which time the curative food is introduced. Since the prefeeding period requires 5 weeks for vitamin A depletion, we have arbitrarily chosen the same period (5 weeks) for the curative feeding, the entire experiment occupying 10 weeks.

Preliminary feeding trials with various amounts of raw milk led us to believe that 1 cc. would produce an average weekly gain approximating the Sherman-Munsell standard of 3 gm. per week. Likewise, we had evidence that 1.5 to 2.5 cc. should be sufficient to bring about a rate of growth approximately equal to the Javillier standard of 6 gm. per week. With these facts in mind the various experimental evaporated milks were fed at 1, 1.5, and 3 cc. levels. In no case were there less than six rats in an experimental group and in several groups the number of experimental animals exceeded this figure, the highest being thirty-one animals in the group receiving 2 cc. of raw milk. The rats were not all

placed on experiment at one time, a number being added to each experimental group from time to time throughout the year. This was done to eliminate seasonal variation influences in case the milk should vary slightly from season to season during the experiment. Males and females were distributed evenly in all groups and, so far as possible, litter mates were distributed in the same manner.

TABLE I.

Average Weekly Growth of Rats Receiving Raw and Evaporated Milk.

Milk fed.	Number of rats in group.	Average weight of rats.		Total gain.	Average gain per wk.	Growth.	Growth injury.
		At start.	After 5 wks.				
cc.		*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
1.0 R. M.	13	63.5	78.3	14.8	2.96	100.00	0.00
1.0 V. E.	8	54.2	63.8	9.6	1.92	64.86	35.14
1.0 V. E. S.	6	53.0	61.3	8.3	1.66	56.08	43.92
1.0 A. E.	6	55.5	61.1	5.6	1.12	37.83	62.17
1.0 A. E. S.	6	52.0	57.1	5.1	1.02	34.45	65.55
1.5 R. M.	14	56.9	83.9	27.0	5.40	100.00	00.00
1.5 V. E.	8	52.5	70.6	18.1	3.62	67.03	32.97
1.5 V. E. S.	9	62.2	80.2	18.0	3.60	66.66	33.34
1.5 A. E.	11	64.2	82.0	17.8	3.56	65.92	34.08
1.5 A. E. S.	10	60.4	77.8	17.4	3.48	64.44	35.56
2.0 R. M.	31	64.2	91.5	27.3	5.46		
2.5 R. M.	10	63.1	94.2	31.2	6.22		
3.0 R.M.	10	63.9	107.3	43.4	8.68	100.00	00.00
3.0 V. E.	9	62.7	99.7	37.0	7 40	85.25	14.75
3.0 V. E. S.	9	63.8	100.8	37.0	7.40	85.25	14.75
3.0 A. E.	7	67.7	104.2	36.5	7.30	84.10	15.90
3.0 A. E. S.	10	74.2	108.0	33.8	6.76	77.88	22.12

When our rats have subsisted on the vitamin A-free basal ration for about 21 days, growth is retarded. By the 28th day, as a rule, growth has ceased and the curve remains flat until the 35th to 37th day when an abrupt fall occurs. At this point the animal will lose from 3 to 6 gm. in a day. It is at this point that we feed the curative food containing vitamin A.

Table I summarizes the data obtained by the curative method. A study of Table I shows that better growth was obtained in every case where raw milk was fed than was obtained after the milk had been evaporated. It will be noted that the average weekly rate of growth on 1 cc. of raw milk (R.M.) was practically equal to the Sherman-Munsell standard. When the milk is evaporated by the vacuum process (V.E.), diluted to its original concentration, and fed at the 1 cc. level in direct comparison with the raw milk, the average weekly growth is 64.86 per cent of that obtained on 1 cc. of raw milk. After sterilization (V.E.S.) by the fractional method described in the first paper of this series, growth is but 56.08 per cent of that obtained with an equal amount of raw milk.

When milk was evaporated by the aeration method (A.E.) growth is but 37.83 per cent and after sterilization (A.E.S.) growth is but 34.45 per cent of that obtained on 1 cc. of raw milk. We are unable to explain the slight difference between the average weekly gains obtained with 1.5 and 2.0 cc. of raw milk (R.M.) in spite of the fact that the groups contained fourteen and thirty-one animals, respectively. At the 2.5 cc. level (R.M.) the average gain per week is practically identical with the Javillier standard of 6 gm. per week.

When the experimental milks were fed at the 1.5 cc. level the same general relationship is noted except that the average weekly growth is better. When the 1.5 cc. (R.M.) growth average is used as the standard the vacuum evaporated (V.E.) group is 67.03 per cent while sterilization (V.E.S.) has reduced the growth slightly, although it is doubtful if such slight differences are significant.

At the 3 cc. feeding level the relation of one group to another is about the same as the ones discussed above with the exception that growth in all groups was much better, which should be expected. It is significant to note that the effect of temperature and aeration becomes less marked at the highest level of feeding while the effects are quite marked in those groups receiving the least amount of milk. It is probable that slight quantitative differences in the vitamin content of foods cannot be determined with the same accuracy by using normal rates of growth as can be obtained with standards of the Sherman-Munsell type. On

the other hand, we have observed a much greater proportion of respiratory troubles and infections of various types when animals are allowed to grow but 3 gm. per week than we have observed in animals growing at the rate of 6 gm. per week.   This is in general agreement with the findings of Macy and coworkers (8), although they use normal growth (12 to 14 gm. per week) as their standard.   We have not been able to obtain the growth response on raw cow's milk obtained by the last mentioned investigators (9), who obtain average weekly growth, by the curative technique, of 12 to 13 gm. per week on 3 cc. of cow's milk, while we obtain weekly gains of 8 to 9 gm. on the same amount of milk.   It is possible that the environment and diet of the cows may account for this difference.   The herd from which our milk was obtained received the same ration the year round, had no access to pasture, and received but a limited and variable amount of green feed during the summer months in the form of green hay.

We have not been able to depend on the onset of ophthalmic symptoms as an index of vitamin A depletion for the reason that many of our animals show no signs of this condition at the time that the body weight becomes stationary, following the prefeeding period.   In fact, an examination of our records shows a much smaller percentage of animals afflicted with xerophthalmia than is reported by many workers.   In this study not more than 30 per cent of our animals showed evidences of the disease.   In the past, when we irradiated the animals directly, we obtained xerophthalmia in about 50 per cent of the cases on record.   As soon as we abandoned direct irradiation in favor of food irradiation, the number of cases fell to about one-half of the former figure.

Postmortem records were kept of all animals and particular attention was paid to ear, lung, and tongue infections.   We have been able to find but one or two cases of tongue infections as described by Macy and coworkers.   In those groups which grew at a rate of 3 gm. or less per week we observed a greater number of cases of ear and lung infection than in the groups which grew at 6 gm. or more per week.   In general, the infections were less severe at the higher growth levels than at the lower levels, although there were a few exceptions to this generalization.

We have expressed, in Table I, the percentage of loss in weight

or failure to grow, using the raw milk groups as standards. We make no attempt at this time to take a definite stand for any of the growth standards discussed. We have the feeling that normal growth standards are not capable of detecting small differences in the vitamin content of foods while we are also somewhat disturbed when we apply the Sherman-Munsell standard for the reason that we are in doubt regarding the influence of lowered resistance and infection on the results. While we are not yet ready to advocate it, we are inclined to favor the "half normal" standard of Javillier, since health is fairly good at that rate of growth and biological response to small food additions seems to us to be fairly well marked.

It would appear that vacuum and aeration methods of evaporation possess an appreciable destructive effect on the vitamin A content of milk. We feel that this destructive effect cannot be due to the effect of heat on the mineral content of the milk for the reason that McCollum's Salt Mixture 185 was fed throughout the experiment. It would appear, therefore, that the loss in nutritive value must be due to destruction of vitamin A. While it is impossible to draw accurate conclusions regarding the amount of vitamin A destroyed it would appear that it may vary from 10 to 30 per cent, depending upon the treatment the milk has received and upon the growth standards used in making the interpretations.

SUMMARY.

Experiments are described in which rats were fed a vitamin A-free, irradiated basal ration, the curative technique being used. Evaporated milks made by vacuum and aeration methods were fed, at various levels, in direct comparison with the raw milk from which they were made. It is concluded that evaporated milks made by the vacuum process have lost some of their nutritive value during the manufacturing process and that this loss, which does not appear to be unduly great, is probably due to destruction of vitamin A. It would appear also that aeration and sterilization increase this destructive effect to some extent. Quantitative interpretations of the destruction of vitamin A are discussed.

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CHANGES IN THE OXYGEN CAPACITY OF THE BLOOD PIGMENT OF RABBITS FOLLOWING SPLENECTOMY.

By BARBARA B. STIMSON.*

(From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York.)

INTRODUCTION.

Much work has been done on the relation of the spleen to the blood in general and the red cell in particular, but one phase of the problem, that bearing on the possible influence of the spleen on the character of the blood pigments, has not been thoroughly explored. Recent work (7) has shown that in dogs the removal of the spleen causes an increase in the non-oxygen-bearing hemoglobin after the administration of nitrobenzene. The possibility that the simple removal of the spleen without the administration of any drug might bring about some alteration in the hemoglobin was thereby suggested and to clear up that point the following work was undertaken.

Methods.

Rabbits were used as experimental animals. All bleedings, usually from 5 to 7 cc. in amount, were made from the ear veins after local dilation with xylene and all blood samples were oxalated. An initial sample was taken 1 or 2 days before operation and further specimens were obtained daily for varying periods afterward. The Van Slyke carbon monoxide method (8) for obtaining the relation between total pigment and oxygen capacity was used in the analysis of all samples, the blood being saturated with New York City illuminating gas. 0.2 cc. of blood was analyzed in each determination and all results were checked within 0.2 volumes per cent. For particulars of the method the reader is referred to Van Slyke's original article.

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* National Research Council Fellow in Medicine.

Spectrophotometric readings were also made on most of the blood samples.   1 cc. of blood was diluted to 100 cc. with a 0.4 per cent solution of ammonium hydroxide (5) and centrifuged for 30 minutes.   Examinations were then made with a Hilger spectrophotometer, the position of the prism being check with a mercury arc line before and after each series of readings.   The light absorption, ϵ, was obtained at each of four wave-lengths, Å. = 6000, 5750, 5600, and 5400, the average of four readings being taken as the recorded value.   Cells of 5 mm. thickness were used to hold the solutions.

The surgical technique of the splenectomy operations was made as simple as possible, care being taken to avoid unnecessary loss of blood.   Ether anesthesia was used in all cases, lasting on an average about 35 minutes.

Results.

Fifteen rabbits were operated upon.   In ten the spleen was removed and in five, used as controls, the abdominal cavity was opened but the spleen was left in place.

The preoperative blood sample in every instance showed a value for the oxygen capacity that agreed within experimental error with the total blood pigment.   In each of the succeeding samples taken from the five control animals there was found no deviation from this agreement.   The loss of total pigment after the operation was closely paralleled by the loss in oxygen capacity.

In the splenectomized animals, however, the drop in oxygen capacity exceeded that in total hemoglobin in the first 2 or 3 days after operation.   The figures shown in Table I represent the difference between oxygen-carrying pigment and total pigment in per cent of the latter.   All samples taken are indicated.

The spectrophotometric readings showed no evidence of methemoglobin at any time as the ratios obtained for the light absorption at two given wave-lengths (Å.= 5400 and 5600) differed not only from those given for methemoglobin in the literature (1, 2, 4) but also from those obtained by the writer for solutions of known methemoglobin content.   The latter were distinctly lower than the ratios for oxyhemoglobin solutions but the ratios obtained for the blood samples after splenectomy were equal to or higher than the oxyhemoglobin values.

TABLE I.

Amount of Non-Oxygen-Bearing Hemoglobin in Per Cent of Total Blood Pigment.

The last five experiments are the controls.

Experi-ment No.	Initial day.	Days after operation.							
		1	2	3	4	5	6	7	12
1	0	4.94	14.73	6.22	0		0		
2	0	4.94	13.98	5.32			4.65		
3	0	6.08	10.70	0		0			
4	0	10.08	20.60	0					
5	0	5.93	9.60	0	0	0			
6	0	7.70	0		0		0		
7	0	4.33	2.75	0	0				
8	0	10.60	4.17	0					
9	0					5.65			0
10	0					11.58			0
11	0	0	0		0			0	
12	0	0	0	0	0				
13	0	0	0	0					
14	0	0	0	0	0				
15	0		0	0	0	0			

DISCUSSION.

From this evidence it appears that the removal of the spleen causes the presence in the blood stream of some form of hemoglobin which is incapable of carrying oxygen but which when a strong reducing agent is added to it becomes able to take on oxygen or carbon monoxide.   This is shown in the method of analysis in which to 1 part of a blood sample a reducing agent is added and to another a small amount of water.   Both are then saturated with carbon monoxide.   The carbon monoxide content of both parts is approximately the same in normal blood, but in the blood of the splenectomized animals the gas content of the part containing the water is less than that in the part with the reducing agent (Table I).   The difference between the two is obviously the expression of the amount of a hemoglobin derivative which is incapable of taking on oxygen or carbon monoxide but which may be transformed into active hemoglobin by a reducing agent.   Conant and Fieser (3) state: "It is apparent that what one is really measuring is the amount of material which by itself has no oxygen

capacity, but which after treatment with a reducing agent combines with oxygen reversibly. As far as is known, *the only substance which has this very peculiar property is methemoglobin."* The substance with which we are dealing has this property in common with methemoglobin but does not show the spectrophotometric picture characteristic of the latter pigment. It seems, therefore, safe to assume that we are dealing with an oxidation product of hemoglobin, similar to but not the same as the accepted form of methemoglobin. Further work is being undertaken to obtain more information, if possible, as to the nature of this pigment.

Because of the part the spleen plays in blood destruction (6) the question arises as to whether or not this non-oxygen-carrying pigment might be some form of partly destroyed pigment normally removed or restored by the spleen. This hemoglobin derivative is, however, capable of being activated, in terms of oxygen capacity, by a strong reducing agent. Might it be possible that the spleen furnishes such a reducing agent which is either secreted into the general circulation, or acts locally while the blood is passing through the organ? Such a possibility has been suggested before (7).

The non-oxygen-carrying pigment need not necessarily be a destruction product of hemoglobin but might possibly be some oxidation product normally prevented from accumulating in the circulation by a reducing action of the spleen in spite of the powerful oxidizing system in the blood.

That the spleen is not the only organ in the body capable of this action is evidenced by the fact that the inactive pigment does not persist in the circulation and has disappeared usually by the 3rd or 4th day after splenectomy. The remainder of the reticuloendothelial system might conceivably take over this function.

The results obtained in this investigation may be summarized as follows: In each of ten splenectomized rabbits a non-oxygen-carrying hemoglobin varying in amount from 4 to 20 per cent of the total blood pigment appeared in the circulating blood after operation. This inactive pigment disappeared in 3 to 6 days. It did not show the characteristic spectrophotometric picture of methemoglobin.

Five control rabbits showed no such inactive pigment.
A reducing action of the spleen is postulated.

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SUGAR AND CHOLESTEROL IN THE BLOOD SERUM AS RELATED TO FASTING.

By RICHARD E. SHOPE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton.)

INTRODUCTION.

In some recent experiments (1) to determine the cholesterol content of the blood serum of normal guinea pigs, variations were noted from week to week which it was difficult to account for on the basis of errors in the method used or changes in the type of diet. It seemed possible that general nutritional conditions might be responsible for these variations and, as a first step in the further study of the question, observations on the changes in cholesterol and its esters in the blood serum during fasting were undertaken.

As the work was being started opportunity presented itself to extend the observations to the human subject and eventually, in order that the general significance of the results might be fixed, other species were included.

Since some recent work, discussed later, points to there being some relationship between the blood sugar and blood cholesterol, parallel determinations on the serum sugar were made.

Bloor (2) has noted that in diabetes mellitus, the hyperglycemia is accompanied by hypercholesteremia. Mahler (3) found that, during ether anesthesia, there was a rise in the blood cholesterol roughly proportional to the rise in blood sugar, and to the duration of anesthesia. Insulin administered several hours before anesthesia prevented the rise in both the blood sugar and blood cholesterol. Shih-Hao and Mills (4) made the observation that in cases of nephrosis showing high blood cholesterol and blood fat but with normal blood sugar values, insulin injections caused a decrease in both the cholesterol and fat of the blood although practically no change in blood sugar was observed. These observations indicate that there may be some relationship between the mechanism controlling the blood sugar level and that controlling the blood cholesterol level.

Data on blood cholesterol values during a complete fast are limited to the following observations. Rothschild (5) using rabbits as his experimental animals noted an irregular although quite marked rise in blood cholesterol during fasting in most of his animals. Ellis and Gardner (6), using rabbits also, noted a rise in both the free cholesterol and ester cholesterol of the blood during a period of fasting.

Available observations on the influence of fasting on the blood sugar follow. Underhill and Hogan (7), in determining the influence of hydrazine on the utilization of dextrose by rabbits, fasted a number of their control animals for 4 days. They state that fasting causes no change in the content of blood sugar. Morgulis and Edwards (8) observed that dogs, fasted until they had lost about 40 per cent in body weight, showed a decrease in the blood sugar level in the early part of the experimental period followed by a rise to the normal level or higher, late in the fast. Allen (9) considered that the regulation of the percentage of sugar in the blood was of such a constant nature normally that it was at most slightly affected by prolonged fasting. Joslin (10) found that, in a human being who was allowed only small amounts of bouillon, the blood sugar dropped from 120 mg. per cent to 90 mg. per cent in 4 days. No data bearing on the effect of complete fasting on the blood sugar level of a human being are available at present.

From previous observations it would then appear that during a fasting period the blood cholesterol usually rises and the blood sugar tends to maintain a constant level. In some species, at least, it appears that there may be a decrease in blood sugar during prolonged fasting followed by a rise. Synchronous observations on blood sugar and blood cholesterol have not been made.

EXPERIMENTAL.

Determinations of the sugar, cholesterol, and cholesterol ester were made on serum rather than on whole blood because the serum content represents more accurately the actual content available for exchange between the blood fluids and either tissue or blood cells. Any possible influence of anticoagulants on the sugar values obtained is eliminated also by using serum rather than whole blood or plasma.

Folin's (11) new method was used for the sugar determinations, Bloor's (12) method for total cholesterol, and Bloor and Knudson's (13) method for the determination of ester cholesterol. The amount of alcohol-ether extractive called for in the last two methods was doubled because a number of animals studied showed extremely low cholesterol and cholesterol ester values. Thus

20 and 40 cc. samples of the alcohol-ether extractive were used in determining total and ester cholesterol, respectively, instead of 10 and 20 cc. as called for in the original methods. In the new Folin method, neutralization of the protein-free filtrates was carried out as recommended.

The blood on which the determinations were made was obtained from the marginal ear vein in the case of the rabbits, from the heart in the case of the cat and guinea pigs, from the tail (arterial) in the case of the swine, and from the median basilic vein in the case of the human. Struggling in the case of the animals bled was minimized as much as possible by gentle handling. It was not noticeably more marked at one bleeding than another so that results obtained are comparable as to the possible error introduced in the sugar values by the factor of struggling. Sugar determinations were made within 2 hours of the time the blood was drawn and the determinations of cholesterol and cholesterol ester were started on the same day that the blood was drawn. In the few cases in which sugar determinations were carried out on whole blood, corpuscles, or plasma, dry powdered sodium citrate was the anticoagulant used.

In so far as slight differences due to species seem to exist, the results obtained will be grouped accordingly and in the following order: human, swine, cat, guinea pig, and rabbit.

A. Human.

A perfectly healthy normal young woman (H.S.) volunteered to undergo the period of fasting required for the experiment. She was 22 years of age, weighed $148\frac{3}{4}$ pounds at the beginning of the experiment, was 5 feet 4 inches in height, and was 6 days postmenstrual at the beginning of the experiment. She led an extremely active life, being an instructor in physical education in a high school. She carried on her work throughout the whole period and her fast was complete as to food. She was allowed all the water she cared to drink. The results of this experiment are given in Table I.

B. Swine.

A castrated male Chester white swine was used. Its weight at the beginning of the experiment was around 120 pounds and

the animal was normal in every apparent way.   The results of this experiment are given in Table II.

TABLE I.

Human.

Date and day of fast.	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.		Whole blood sugar.	Weight.	Urine pH.	Acetone bodies in urine.*	Remarks.
1927	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*mg. per cent*	*. lbs.*			
Mar. 1, 7.30 a.m...	109.8	231.4	171	73.8		148.75	6.3	0	7.00 a.m. 1 cup cocoa, 2 slices buttered toast.
Mar. 2, 8.15 a.m., end of 1st day.	87.8	256.4	183	71.3		146	6.3	+	
Mar. 3, 8.15 a.m., end of 2nd day.	66.6	245.1	186	75.8		144	5.7	+++	
Mar. 4, 7.30 a.m., end of 3rd day..	44.4	287.3	196.5	68.4		140.75	5.5	++	
Mar. 5, 5.30 a.m., end of 4th day.	37	308.6	196.5	63.6		138.5	5.3	+	
Mar. 6, 6.00 a.m., end of 5th day.	68	314.4	255.3	81.2	67.3	137.5	5.5	+	
p.m. 1.30..........	53.2	293.9	263.7	89.7	45.4				
1.45..........	Fed juice of 2½ oranges to which had been added 30 gm. cane sugar. 2 slices toast, buttered and sugared.								
2.45..........	235.3	252.5	217		85.9	196			
5.45..........	101	277.7	215.6	77.6	109.8		6.0	+	Urine passed at 8.15 p.m.
8.45..........	72.7	277.7	231.5	83.3	83.3				

' Lange's method.   + = Slight to moderate reaction.
++ = Marked reaction.
+++ = Very marked reaction.

C. Cat.

The cat used was a young male weighing about 1.5 kilos. It was apparently normal in every way. The results obtained are given in Table III.

TABLE II.
Swine.

Date and day of fast.	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.		Remarks.
1927	*mg. per cent*	*mg. per cent*	*mg. per cent*	*per cent*	
Feb. 14, 11.00 a.m...	130.4	125	75.3	60.2	9.30 a.m., fed corn, mangels, and cracked corn, bran, middlings mixture. 11.00 a.m., all food removed from cage. Water *ad libitum*.
Feb. 15, 10.00 a.m., end of 1st day.....	105.6	134.4	86.8	64.5	
Feb. 16, 10.00 a.m., end of 2nd day....	83.8	148.8	97.6	65.5	
Feb. 17, 10.00 a.m., end of 3rd day.....	121.8	154.3	100.8	65.3	
Feb. 18, 6.30 a.m., end of 4th day.....	107.1	154.3	104.1	67.4	
6.40 a.m.........	Fed mixture of cracked corn, bran, middlings, meat, and bone meal. Allowed to eat all desired for ½ hr., remaining food removed.				
7.40 a.m.........	214.2	148.8	88	59.1	
9.10 "	245.9	137.3	81.6	59.4	
10.40 "	267.8	143.6	89.3	62.1	
12.10 p.m	211.2	152.4	102.4	67.1	
1.40 "	133.9	151.6	90.6	59.8	
3.10 "	121.9	154.3	99.2	64.2	
4.30 "	125	154.3	111.6	72.3	

D. Guinea Pigs.

Because of the small amount of blood that can be taken repeatedly at frequent intervals from a single guinea pig each

determination was made on groups of forty animals. 1 cc. of blood was drawn from the heart of each animal and the 40 cc. thus obtained were pooled and the sugar and cholesterol determinations run on this as a single sample. The blood, as nearly as

TABLE III.

Cat.

	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.		Remarks.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
Normal....... ...	129.3	152.4	101.4	66.5	Fed bread and milk 2 hrs. previous to this bleeding.
After 42 hrs. fasting.	110.2	178.5	120.2	67.3	

Fed bread, milk, and 1 white mouse.

	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.	
2 hrs. after feeding.	129.3	158.2	94.7	59.8

TABLE IV.

Guinea Pigs.

	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.		Remarks.
	mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
Normal.............	137.1	42.8	23.1	53.9	Fed mangels, oats, and hay 2 hrs. previous to this bleeding.
After 42 hrs. fasting...........	107.5	57.2	30.9	54	

Fed mangels, allowed to eat for 30 min.

	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.	
2 hrs. after feeding.	199.3	50.9	24.9	48.9

could be determined from its color, was from the right heart. None of the forty animals bled was killed during the three bleedings so that the three samples were identical and complete as to animals represented. The results obtained are given in Table IV.

TABLE V.
Rabbits.

Animal No.	Time in period of fast.	Serum sugar.	Total serum cholesterol.	Serum cholesterol bound as ester.		Remarks.
		mg. per cent	*mg. per cent*	*mg. per cent*	*per cent*	
57	Normal.	166.6	49	23	46.9	All animals fed cabbage, oats, and hay 2 hrs. previous to normal bleeding.
	After 66 hrs. fasting.	172.4	90.9	58.4	64.2	
	Fed cabbage, allowed to eat 30 min.					
	2 hrs. after feeding.	148.5	74.4	41.6	55.9	
58	Normal.	141.5	38.6	19.4	50.3	
	After 66 hrs. fasting.	164.8	73.5	49.9	67.8	
	Fed cabbage, allowed to eat 30 min.					
	2 hrs. after feeding.	185.1	64.4	36.3	56.3	
59	Normal.	115.3	43.7	29.9	68.4	
	After 66 hrs. fasting.	145.6	74.4	43.4	58.3	
	Fed cabbage, allowed to eat 30 min.					
	2 hrs. after feeding.	145.6	55.3	38.5	69.6	
60	Normal.	88.8	61.0	31.8	52.1	
	After 15 hrs. fasting.	139.8	74.0	30.4	41.0	
	After 40 hrs. fasting.	143.9	103.5	55.1	53.2	
	After 63 hrs. fasting.	122.7	152.9	66.6	43.5	
	Fed cabbage, allowed to eat 30 min.					
	1 hr. after feeding.	198.0	137.7	81.6	59.2	
	3 hrs. after feeding.	162.6	126.2	52.7	41.7	
	5½ hrs. after feeding.	145.9	108.9	44.3	40.6	

E. Rabbits.

Because rabbits appeared to differ in certain aspects of their behavior towards fasting from that shown by other species studied, a number of animals was used, all of which verified this difference. Male animals were used throughout. Two control animals which were put through the régime of bleeding but which were not fasted showed no significant variations in serum sugar, total cholesterol, or cholesterol ester values throughout the experimental period. The results obtained with rabbits are given in Table V. Because of the nature of the results obtained in these

TABLE VI.

Rabbit 61 (1.2 Kilos).

·Time in period of fasting.	Serum sugar.	Total serum choles-terol.	Serum choles-terol bound as ester.		Remarks.
	mg. per cent	*mg.* per cent	*mg.* per cent	*per cent*	
Normal..............	128.0	73.7	38.4	52.1	Fed cabbage, oats, and
After 15 hrs. fasting..	157.4	81.7	36.5	44.6	hay 2 hrs. previous to
" 40 " " ..	127.4	99.8	53.1	53.2	normal bleeding.
Insulin, Lilly, 20 units subcutaneously.					
1 hr. after insulin....	35.4	89.1	56.4	63.3	Convulsions in 2½ hrs. after administration of insulin.
6½ hrs. " "	15.7	85.4	41.6	48.7	Bled from heart. Animal moribund.

experiments with rabbits one experiment was conducted in which, instead of feeding the animal at the termination of the fasting period, 20 units of insulin were administered subcutaneously. The results of this experiment are given in Table VI.

DISCUSSION OF RESULTS.

Three salient points were brought out by the experiment on the human being and were verified by subsequent experiments with laboratory animals. The observations are summarized as follows: first, there is a variation in total cholesterol and cholesterol bound as ester during fasting; second, there is a variation in serum

sugar during fasting; and third, it appears that there may be a relationship between the variations in serum sugar and serum cholesterol during fasting.

1. Variation in Cholesterol and Cholesterol Ester.—In accord with the results of Rothschild (5) and Ellis and Gardner (6) on whole blood, a quite marked rise in serum cholesterol was found to take place during fasting. This rise was evident within 24 hours of the beginning of the fasting period and persisted throughout with a steady though irregular elevation. An immediate drop occurred with the intake of food terminating the fast. This drop was followed by a second rise as the food eaten was utilized and the subjects were again in a fasting condition.

Cholesterol bound as ester rose, during a period of fasting, in a roughly parallel fashion to the rise shown by total cholesterol. Following the taking of food at the termination of the fast the cholesterol bound as ester dropped at a more rapid rate than did the total cholesterol. Rabbits formed an exception, in that, at about an hour after feeding, cholesterol ester on the blood serum was found to be increased. This, however, was followed by the drop observed in other species.

2. Variation in Serum Sugar.—In the human subject the serum sugar was observed to drop in a regular and uninterrupted progression until the end of the 5th day of the fast when a rise to a level higher than that at the end of the 2nd day was observed. By the end of the 4th day it had reached the very low level of 37 mg. per cent. That this low level probably was true of the whole blood as well as the blood serum is suggested by a comparison of serum and whole blood sugar determinations conducted on the following 2 days in which the serum sugar concentration was found to be equal to or greater than that for the whole blood. Serum sugar decreased as a result of fasting in the swine, cat, and guinea pigs also. The swine showed a rise in serum sugar following the preliminary drop. Rabbits formed a marked exception in that their serum sugar level increased during the fast period and was usually still above the normal value after 60 hours without food.

3. Relationship between Serum Sugar and Serum Cholesterol.— Reiterating what has been said in the two preceding paragraphs, we note that a relationship between cholesterol and sugar in the

blood serum exists during a period of fasting and following feeding at the termination of the fast. As the serum sugar decreased throughout the fast periods there was a steady increase in both total and ester cholesterol. The increase in cholesterol and its esters was most marked late in the fast and here the rise was largely due to the increase in ester cholesterol. Following feeding at the termination of the fast, when the serum sugar rose rapidly and abruptly, the total cholesterol and cholesterol ester dropped as abruptly with ester cholesterol showing a more rapid drop than free cholesterol.

The significance of the figures obtained in the case of the rabbit has been determined by a member of the departmental staff on the basis of the probable errors involved in the experiment. The chances in favor of the figures being significant are as follows:

(a) Serum sugar rise in fasting........................... 20:1
(b)   "   cholesterol rise in fasting...................... 50:1
(c)   "   "   ester rise in fasting................<100:1
(d)   "   "   drop at end of fast.................. <50:1
(e)   "   "   ester drop at end of fast............ 50:1

The rise in serum sugar found 2 hours after the end of the fast was not significant, nor were the variations in the percentage of cholesterol bound as ester.

The experiments with species other than the rabbit, being concerned with single individuals, do not so readily lend themselves to mathematical treatment.

The low serum sugar concentrations found during the period of the fast in the case of the human being require some consideration because these low values are contrary to the only pertinent statements found in the literature. It is very possible that the active life led by H.S. during her fast might explain, in part, the very low values obtained. If the matter of exercise did influence the serum sugar it exerted a cumulative rather than an immediate effect for the patient was bled each time in the morning soon after rising. Rakestraw (14) in determining the effect of exercise on blood sugar values noted a decrease in plasma sugar only after prolonged rather violent exercise. Short periods of exercise caused a rise in plasma sugar. It is possible that the greater delicacy and accuracy of the new Folin method may account for the lower values just described.

It is of interest to note that during the experimental period the patient was at times well within the reported "insulin shock" range as regards degree of hypoglycemia and still suffered from none of the symptoms commonly associated with this phenomenon.

Instead of remaining at the low level reached at the end of the 4th day the serum sugar rose on the 5th day to approximately the level of the 2nd day of the fasting period. This rise cannot be explained on the basis of the data presented, and it is probably not analogous to the rise described by Morgulis and Edwards (8) as occurring in dogs after they have lost about 40 per cent in body weight. It is doubtful whether the rise represents an actual increase in carbohydrate being metabolized. It certainly is not associated with changes in the concentration of cholesterol ester in the blood serum that would be expected to result from an increase in the utilization of carbohydrate in the light of what takes place following the intake of food at the termination of the fast.

The results obtained with rabbits call for special consideration. So far as cholesterol and its esters are concerned the rabbit behaves practically as do the other species. It is of interest to note that Rabbit 61 (Table VI) on being given insulin, showed a drop in total cholesterol equivalent to that seen in Rabbit 60 (Table V) 1 hour after feeding.

The serum sugar, contrary to its behavior in the other species, rises in the fasting rabbit. In conformity to the others, feeding immediately and markedly increases its amount. The actual amount of sugar present in the blood at any moment must represent a balance between income from its various sources and concurrent consumption. It is possible that the fasting rabbit goes very quickly to a slower rate of carbohydrate utilization.

These observations on the rabbit show a suggestive similarity to those reported by Bloor (2) in diabetes and by Mahler (3) in ether anesthesia, both conditions being characterized by a high content of the blood in both sugar and cholesterol.

In general, the immediate way in which changes in the sugar are associated with those of the cholesterol of the serum, throughout the fasting period and consecutive to the taking of food at its termination, strongly suggest that at some point a common factor is involved. Since it is established that the metabolism during

the early days of a fast is marked by an increasing dependence on the stored fat of the body, and since it is difficult to see any point of direct contact between cholesterol and the processes of carbohydrate metabolism, so far as those are understood, it is rational to think of the observations as being in accord with the views of Bloor and others (2, 15, 16) that the cholesterol of the blood is in some way functional with relation to the metabolism of fat.

Any effort to fix more precisely the function of the blood cholesterol at once encounters difficulties due to obvious lack of essential data. It may be regarded as significant in this connection however, that the present work has clearly shown that the quantitative stability of blood cholesterol, until recently generally assumed, not only does not prevail over long periods but does not even hold from hour to hour in the face of rather simple alterations in the metabolic state of the animal. The way is clearly opened to further experimentation designed to fix the place of the cholesterol and cholesterol esters of the blood and active tissues in the general metabolism.

SUMMARY AND CONCLUSIONS.

1. During a period of fasting there is a decrease in the sugar content of human, swine, cat, and guinea pig blood serum with an increase in both total cholesterol and that bound as ester.

2. Feeding at the termination of a fasting period produces in the human, swine, cat, and guinea pig a rise in the sugar content of the blood serum and a drop in the total cholesterol and cholesterol ester content.

3. The sugar content of human blood serum may reach a very low level during fasting.

4. The low level in blood serum sugar is followed by a secondary rise, variable in extent and time relationship with species.

5. In the rabbit during a period of fasting there is a continuous rise in the sugar, total cholesterol, and cholesterol ester content of blood serum. Following feeding at the termination of the fast period, the serum cholesterol and cholesterol ester drop as in the other species of animals studied.

6. Insulin, administered to a fasting rabbit, has the same effect on the cholesterol and cholesterol ester content of the blood serum as does feeding a meal high in carbohydrate.

7. The correlative behavior, generally inverse, between the serum sugar on the one hand and the serum cholesterol and cholesterol esters on the other, through a period of fasting and the subsequent feeding lends weight to the view that cholesterol and its esters function in some direct way in the transport or metabolism of fat.

Addendum.—Since this work has been completed my attention has been called to a paper by Lennox, W. J., O'Connor, M., and Bellinger, M., *Arch. Int. Med.*, 1926, xxxviii, 553, and *Arch. Path. and Lab. Med.*, 1927, iii, 834, in which data for the human subject, similar in a number of respects to that given in this paper, were published.

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ON THE SEPARATION OF HISTIDINE AND ARGININE.

III. THE PREPARATION OF ARGININE.*

By HUBERT BRADFORD VICKERY AND CHARLES S. LEAVENWORTH.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

Two fundamentally different methods have been employed for the preparation of arginine, both of which were originated by Kossel and his collaborators. The more recent of these, which depends upon the precipitation of arginine from a protein hydrolysate by means of 2,4-dinitro-1-naphthol-7-sulfonic acid (1) has been modified and improved by Pratt (2). In the present communication we describe modifications of the earlier Kossel procedure which permit the preparation of arginine in an expeditious and relatively easy manner.

We have recently (3) shown that histidine may be quantitatively separated from arginine by the precipitation of its silver compound with barium hydroxide at pH 7.0, and that the arginine may be recovered from the filtrate by precipitation as its silver compound with barium hydroxide at a reaction in the range pH 10 to 11. This technique may be advantageously used for the preparation of arginine in quantity, since the precipitation of arginine silver is quantitative and the recovery of the arginine, either as crude carbonate or as pure crystallized picrate, is readily effected. Moreover the conversion of the pure picrate to carbonate is less troublesome than the recovery of arginine from the dinitronaphtholsulfonate.

The method we have employed may be indicated most briefly as follows:

1. The protein (edestin) is hydrolyzed and the greater part of the acid removed.

2. An excess of silver oxide suspended in water is added; the acidity of the solution is maintained by the addition of dilute sulfuric acid as required.

3. The solution is brought to pH 7.0 by the addition of cold saturated barium hydroxide solution, and the voluminous precipitate, which contains the histidine together with other amino acids, is removed and washed with water.

4. The filtrate is acidified to Congo paper with sulfuric acid and again treated with silver oxide until excess is present;[1] hot saturated barium hydroxide is then added until the reaction is strongly alkaline to phenolphthalein. The precipitate contains little except silver oxide, barium sulfate, and the silver compound of arginine. It is removed and washed with water made alkaline with barium hydroxide.

5. The precipitate is suspended in water, sufficient dilute sulfuric acid added to give the faintest possible reaction to Congo paper, and treated with hydrogen sulfide. Silver sulfide is removed, the solution concentrated slightly to remove hydrogen sulfide, and treated with the requisite amount of a solution of recrystallized barium hydroxide to precipitate the sulfuric acid exactly. The barium sulfate is removed and washed, and the solution concentrated *in vacuo* to a thin sirup. Free arginine will usually crystallize on scratching the walls of the flask.

6. If further purification is required this material is converted to the picrate which may be recrystallized and the arginine recovered as carbonate (3).

The operations up to step (6) may be readily completed in 5 working days on 250 gm. of protein if the hydrolysis is performed in an autoclave and a large centrifuge is used to remove precipitates.

Discussion of the Procedure.

Edestin is best prepared by the procedure described by Osborne, Wakeman, and Ferry (4). Units of 30 pounds (13.6 kilos) of hemp seed are worked up and the yield of air-dry protein is usually

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[1] For the explanation of this step see below.

in the vicinity of 1 kilo.   The preparation is very easy if proper facilities are available.   Failing edestin, gelatin is probably the most suitable protein.   It is important to note that a relatively pure preparation of protein should be used.   Crude preparations give much trouble owing to the reduction of the silver salts.

The hydrolysis may be effected by boiling the protein with 20 per cent hydrochloric acid or 33 per cent by volume sulfuric acid for 24 to 30 hours.   If time is a factor the protein is best hydrolyzed by treatment with 5 N sulfuric acid for 5 hours at 150°C.   Although this method undoubtedly leads to some secondary decomposition as shown by the higher proportion of ammonia in the hydrolysate, the yield of arginine is scarcely affected.

Hydrolysis of proteins in an autoclave has frequently attracted attention and has been studied in some detail by Henriques and Gjaldbäk (5) and by Greenberg and Buck (6).   These authors have established conditions under which 4 per cent and 1 per cent solutions of certain proteins respectively, may be hydrolyzed completely.   Unfortunately, however, the conditions for the complete hydrolysis of more concentrated solutions of proteins at higher temperatures have not been investigated and in practical work it is these higher concentrations which must be employed if considerable quantities of protein are to be treated in laboratory size apparatus.   This problem requires more study than we have as yet had opportunity to give it.   We can only say at present that hydrolysates obtained as described in the experimental part of this paper have given satisfactory results for the preparation of arginine.

For most purposes hydrochloric acid will generally be employed.   The greater part of this may be removed by distillation and the rest by means of silver oxide in the presence of a little sulfuric acid.   The silver is readily recovered by the procedure described below.

Silver oxide in suspension is an extremely useful reagent.   As employed here (see Kiesel (7)) it is equivalent to the use of silver sulfate but avoids the large volumes of solvent that are necessary when that salt is used.

The precipitate thrown down by silver and barium hydroxide at pH 7.0 is very complex.   It contains all of the histidine and a part, at any rate, of the glutaminic and aspartic acids, together

with tyrosine and other amino acids. The removal of these silver salts usually leaves the filtrate depleted of its excess of silver. This must therefore be renewed by acidification and addition of silver oxide. The precipitation of arginine by the addition of barium hydroxide to a strong alkaline reaction to phenolphthalein is then quantitative and the product is well over 90 per cent pure. We have occasionally encountered difficulty in obtaining the spot test for the presence of excess silver when working with unusually concentrated solutions. Any doubt may be set at rest by diluting a sample of the clear fluid and adding a little silver sulfate solution. If excess is then present the buff-colored precipitate with a drop of barium hydroxide will show up readily.

If silver nitrate is used instead of silver oxide the precipitate of arginine silver must be washed until free from nitrate ion.

Acidification of the arginine silver precipitate with sulfuric acid is necessary to permit the removal of silver sulfide under advantageous conditions. It also removes traces of barium retained by the somewhat flocculent precipitate. Decomposition with hydrogen sulfide is most rapidly accomplished by passing the gas into the suspension during mechanical stirring or shaking.

The exact removal of sulfuric acid with pure barium hydroxide is quicker than the procedure usually recommended of adding excess of barium hydroxide and subsequently saturating the solution with carbon dioxide. The filtrate from the barium carbonate frequently contains much barium, possibly as the barium salt of a carbamate, which separates as carbonate during the concentration. In the procedure recommended barium hydroxide solution is added until 5 cc. samples, when centrifuged clear, give no precipitate on the addition of a drop either of 1 per cent sulfuric acid or of barium hydroxide solution.

EXPERIMENTAL.

Hydrolysis.—500 gm. of edestin were treated with 2500 cc. of 5 N sulfuric acid in a 4000 cc. beaker and stirred until the entire quantity of protein was moistened. The mixture was then heated in an autoclave at 150–155°C. for 5 hours. A small sample of the fluid was removed for analysis and the volume of the remainder

measured so as to permit the calculation of the total quantity of protein present from the nitrogen. The fluid was diluted with an approximately equal volume of hot water and a hot concentrated solution of barium hydroxide was slowly added with constant stirring until the reaction was only faintly acid to Congo paper. The barium sulfate, together with the humus, was then centrifuged off and the fluid concentrated by boiling in large porcelain dishes. The barium sulfate should be washed if the highest obtainable yield of arginine is sought. This is best done by whipping it up with boiling water in a large dish and centrifuging. As much as 6 per cent more of the nitrogen may be recovered by washing only once.

In the actual experimental work under discussion quantities of hydrolysate representing approximately 250 gm. of edestin were used and several modifications of the procedure were tried, such as the use of silver nitrate, the precipitation of histidine and arginine together followed by the separation as silver compounds, etc. The following details therefore apply to different actual experiments which were selected as representing the most convenient procedure for the rapid preparation of pure arginine.

Preparation of the Arginine Fraction.—An excess of silver oxide as a thick suspension in water was added with constant stirring to the hydrolysate at a concentration of from 40 to 50 gm. of protein per liter. The solution was maintained distinctly acid to Congo paper by the addition of dilute sulfuric acid as necessary. Cold saturated barium hydroxide solution was added until the reaction was neutral or faintly alkaline to sensitive litmus paper and further additions then made until a sample of the clear fluid, on testing with brom-thymol blue, gave the same color as a buffer solution of pH 7.0. Considerable time can be saved in this operation if a drop of the indicator is allowed to fall on the surface of the slowly rotating solution. If the transient color produced is yellow, insufficient barium hydroxide has been added; if blue, too much. The correct point to begin more accurate testing is that at which the color is a pale green. When the reaction is correctly adjusted the precipitate settles rapidly. It was removed by centrifuging, washed with water, and then treated with hydrochloric acid for recovery of the silver.

The clear filtrate was acidified to Congo paper with sulfuric

acid and silver oxide added until excess was present. Warm saturated barium hydroxide solution was then added until the solution was strongly alkaline to phenolphthalein. The precipitate was centrifuged off and thoroughly washed with water made alkal ne with barium hydroxide. The easiest and most effective method of washing consists in stirring up the contents of each centrifuge bottle with wash fluid, inserting a stopper, and shaking violently. The thin pulp is then poured through silk bolting cloth and lumps reduced with a brush. This process should be carried out twice. The precipitate was then suspended in water, dilute sulfuric acid added until the reaction was very faintly acid to Congo paper, and the material transferred to a large filter flask equipped with an inlet tube for hydrogen sulfide. The flask was placed on the shaking machine and agitated, while passing the gas, for several hours. The silver sulfide was then centrifuged off and washed. The solution and washings were concentrated *in vacuo* and nitrogen determined to serve as a basis for the subsequent addition of picric acid. The remainder of the solution was freed from sulfuric acid by the addition of the necessary amount of recrystall zed barium hydroxide, centrifuged, the barium sulfate thoroughly washed, and the solution concentrated to a thin sirup. Crystallization was readily induced and the entire sirup soon solidified.

An arginine fraction obtained in this way from 227 gm. of edestin contained 11.27 gm. of nitrogen, which is equivalent to 35.02 gm. of arginine or 15.2 per cent of the protein. This was treated, at a volume of approximately 500 cc., with the calculated quantity (45.9 gm.) of picric acid in 500 cc. of hot alcohol. On stirring, the picrate rapidly separated in small needles which, when dried at 110°C., weighed 64.9 gm. and melted at 217–218°. On evaporation of the mother liquor a second crop weighing 9.6 gm. was obtained.

Picric acid was removed from the mother liquor of this crop and 1.51 gm. of arginine dinitronaptholsulfonate were subsequently obtained from it. These three crops of crystals account for 10.58 gm. of arginine nitrogen in the arginine fraction when corrected for the small aliquot removed for nitrogen determination, or 93.9 per cent of the total nitrogen of this fraction. This is equivalent to 32.86 gm. of free arginine or 14.45 per cent of the

edestin used, a yield slightly higher than either result obtained by Kossel and Patten (8) (13.97, 14.36 per cent).

Recovery of Silver.—As the procedure described above involves the use of liberal quantities of silver salts, the expense is a serious factor unless steps are taken to recover the silver for subsequent use. This may be conveniently done as follows.

The silver sulfide is suspended in water, treated with concentrated nitric acid in small quantities at a time, and simmered gently with occasional additions of nitric acid, until oxidation is complete as shown by the cessation of brown fumes. The insoluble material should be white. Sodium hydroxide is then added until the fluid is only faintly acid, followed by a large volume of concentrated ammonia to dissolve the silver sulfate. At this point silver chloride residues may be added in the form of a thin pulp. The solution is centrifuged, or filtered, from the barium sulfate, brought nearly to boiling, and a strong hot solution of invert sugar or glucose added. Reduction is complete when the silver settles leaving a clear brown solution. This is poured off and the silver washed by decantation, any tendency to go into colloidal suspension being readily prevented by the addition of a little sodium chloride to the wash water. The final washing is carried out with approximately 20 per cent alcohol. The silver is finally sucked out on a Buchner funnel, dried in the oven, heated in a quartz dish until all organic impurities have been driven off, cooled, and dissolved in dilute nitric acid and the solution, if necessary, filtered. Concentrated sodium hydroxide solution is then added in excess and the silver oxide washed nitrate-free by decantation. It may be preserved under water until required and appears to be perfectly stable.

SUMMARY.

Modifications of the Kossel and Kutscher silver procedure are described which permit the preparation of arginine in quantity with the expenditure of the minimum effort and time, and make this base one of the more easily available amino acids.

Silver oxide is recommended as a convenient substitute for the nitrate.

A procedure for the recovery of silver sulfide and chloride is described.

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THE RELATION OF INORGANIC IRON TO NUTRITIONAL ANEMIA.

BY HELEN S. MITCHELL AND MARGERY VAUGHN.

(From the Nutrition Research Laboratory, Battle Creek Sanitarium and College, Battle Creek.)

In a previous publication (1) a method was reported for producing a true nutritional anemia in young rats and promoting recovery by the addition of small amounts of iron from various sources. The four inorganic salts used in this earlier series of experiments showed such interesting results that our attention was directed to further studies on so called inorganic compounds which might be or have been used in the treatment of chlorosis or anemia. The present paper reports a survey of the physiological availability of a number of the least astringent iron salts of widely varying properties. Conclusions from the earlier surveys of the availability of iron salts in man reported by Kletzinsky in 1854, reviewed and confirmed by Bunge (2), are contrary to the present observations on animals.

The experimental procedure in general was the same as that previously reported with a few minor changes for the purpose of improving our technique. The difficulty of raising young anemic rats to the age of 5 or 6 weeks was not entirely overcome by the addition to the basal diet of minerals as suggested by Daniels and Hutton (3). Reproduction and lactation were both below normal in our anemic colony. Sure's report (4) that vitamin E or a similar substance in wheat germ not only increased fertility but also improved lactation suggested the addition of small amounts of wheat germ to the fresh whole milk which has been the basal diet in all our anemia work. It was recognized that the additional vitamin B thus supplied might also be helpful in lactation. There was apparently not enough iron in the 2.5 gm. of wheat germ fed each mother rat per day to cause any

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significant change in the blood picture of the young nursing rats.   The hemoglobin ranged from 2 to 6 gm. per 100 cc. of blood, averaging 3.9 gm. for all rats when started.   Nevertheless, with wheat germ additions there was a marked increase in the number of litters, the number of rats in a litter, and the survival of the young, all of which contributed to the success of our experiments.

Hemoglobin determinations were made weekly by the acid hematin method with a Newcomer disc standard.   All readings have been calculated to the Dare standard previously used, *i.e.* 100 per cent = 13.77 gm. of hemoglobin per 100 cc. of blood, and charts have been drawn on this basis for convenience.   If for any reason a free flowing drop of blood could not be obtained due to inflammation or soreness of the tail, no hemoglobin figure was recorded for that week.

Erythrocyte counts were taken about every 6 weeks or when a change in diet was to be made.   The hemoglobin figures, however, were used as the chief criteria in drawing conclusions because the quantity of hemoglobin seems more significant than the number of erythrocytes in nutritional anemia.   Incidentally we have had an experienced technician examine stained slides of blood smears from most of the animals for any abnormal red cells, but the findings have been negative except in a very few cases which were considered insignificant.

Growth records have been kept on all animals, but these seemed to be significant only in so far as severe anemia might not develop if growth were greatly stunted.   Gains in body weight do not seem to bear any relation to hemoglobin response as indicated in Table I.   Some animals used for test purposes have been slightly underweight when started but have shown a fairly normal rate of growth throughout the experimental period.   Long continued anemia ultimately retards growth as evidenced by a gain of only 80 gm. in 20 weeks in two extremely anemic female rats, as compared with 140 gm. in normal females.

The quantity of iron supplement has been carefully regulated, starting with a small amount and increasing at intervals as indicated on the charts.   Two control rats from each litter received no iron supplement for several weeks, after which they were allowed to recover if possible when supplementary iron was sup-

plied, while the other rats were given a source of iron at weaning. Five to ten rats were used for each compound investigated and more if there was any uncertainty or wide variation in results. Records of a few representative rats from each group have been given in the hemoglobin charts. All the compounds used were of standard quality Merck unless otherwise designated and were used without further purification. Soluble iron salts were fed in solutions which were made fresh every 2 or 3 days or oftener if they showed signs of deterioration. The use of heavy sucrose syrup as a diluent in some instances helped to stabilize the solution, especially the ferrous compounds. Insoluble iron salts were incorporated quantitatively in a starch paste and aliquot portions were fed daily. By this means complete consumption was insured far better than when a small amount of insoluble material was allowed to sink to the bottom of the food cup. Rats liked the starch paste and consumed it promptly and completely. This supplementary carbohydrate *per se* did not seem to bear a significant relation to hemoglobin synthesis since neither the starch nor the sucrose used showed any effect upon the availability of the iron.

The increase in the amount of iron supplement was made periodically in order to test the possibility of greater availability of larger quantities which has been suggested by several European clinicians. It also seems possible that a larger rat still rapidly growing might need an increased supply of iron. In some cases the change to a larger quantity was made following a period of poor response while in others marked improvement had already been obtained, but more was added to see if a still greater response was possib'e. The usual procedure was to start the rats on the 0.4 mg. of supplementary iron, increase this to 0.8 mg. at the end of 6 to 9 weeks, and again double the quantity to 1.6 mg. after several weeks. The salts investigated demand individual consideration as to results obtained.

Two of the compounds (ferric oxide and ferrous carbonate) mentioned in our previous publication (1) as poor in availability were reinvestigated in comparison with modified forms of the same salts commonly used by clinicians. The saccharated and peptonized forms of ferric oxide, both soluble compounds, showed considerably better availability than the insoluble iron oxide
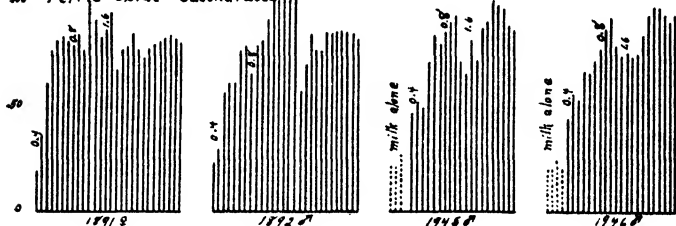
previously investigated.   The response to 0.4 mg. of Fe from the ordinary oxide[1] has been slow and very irregular (Chart I), the

Weekly Hemoglobin Determinations
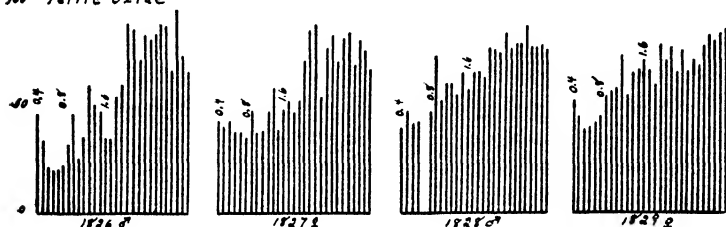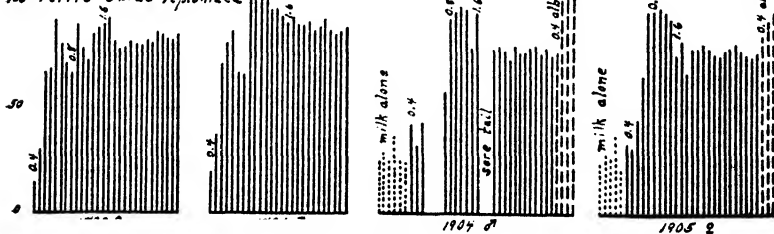
100% = 13.77 grams of hemoglobin per 100 cc. of blood

0.4, 0.8, 1.6 = mg. of Fe from respective sources

CHART I.

average gain in hemoglobin in 6 weeks being 3.5 gm. per 100 cc. Several animals receiving it have died within the first few weeks

[1] Central Scientific Company, Chicago.

of the experiment.   When the quantity fed had been increased
to as high as 1.6 mg. of Fe daily a marked improvement was noted,
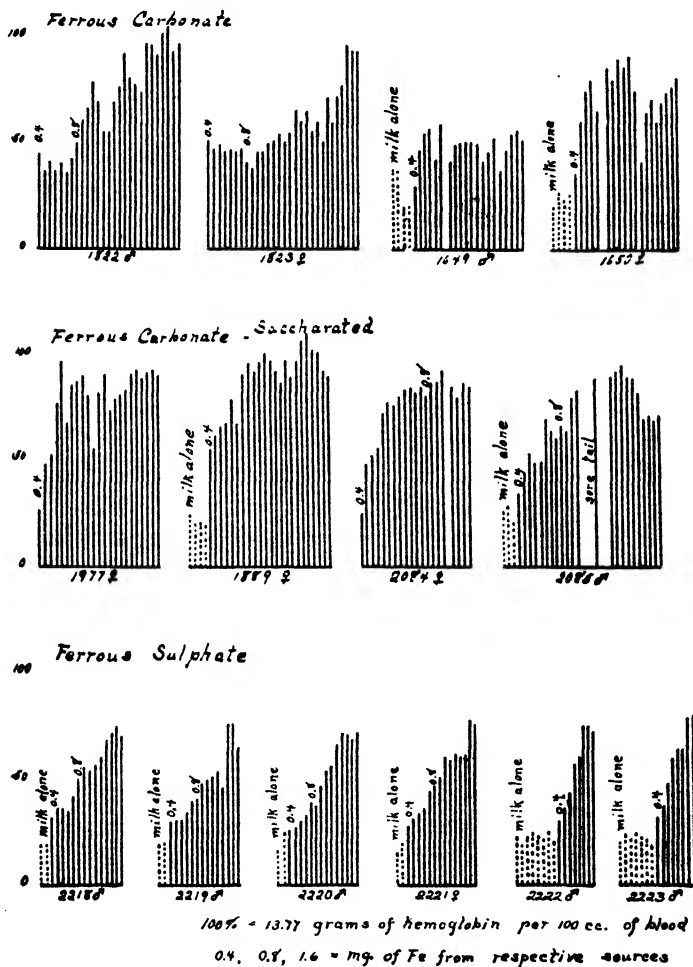

Weekly Hemoglobin Determinations

CHART II.

indicating probably that a small amount of the oxide was utilized
and with a large enough quantity the response became significant.
When these large quantities were fed the feces were reddish in

color, showing that a large amount was excreted unchanged. Fecal analyses on these animals would have been interesting but facilities were not available. The increase in hemoglobin on the saccharated and peptonized forms of ferric oxide was quicker and more consistent, averaging respectively 7 and 6.9 gm. per 100 cc. of blood in 6 weeks with no apparent loss of iron in the feces as far as gross observations showed.

The saccharated ferrous carbonate proved to be somewhat more available to the animal than the simple ferrous carbonate as used in Blaud's pills[2] (Chart II). The saccharated form however, did not stimulate as rapid or as complete a recovery as some of the other iron salts tried.

The ferrous sulfate used was in bluish green efflorescent crystals and was not purified before using. The 2 mg. of the salt necessary to furnish the daily allowance of 0.4 mg. of Fe stimulated only a moderate response in the four rats first used. In 5 weeks the hemoglobin increased only about 3 gm. (Chart II). Upon doubling the quantity a somewhat better response was observed but still far below the maximum possible with some other forms of iron. Two rats which had been on the milk without iron supplement for 7 and 8 weeks showed a more rapid response than the previous group when the ferrous sulfate was given. These ferrous sulfate tests were started later and are therefore of shorter duration than most of the others.

Ferrous iodide was used as a solution put up in ampules[2] to be prepared for use in a heavy sucrose syrup commonly known as saccharated ferrous iodide or the syrup of ferrous iodide. When 0.4 mg. of Fe daily was furnished from this source there was an immediate and fairly rapid rise in hemoglobin, showing an average increase of about 6 gm. in 4 weeks. This response was not always permanent, however, as will be noted from Chart III. With an increase in quantity fed there was usually a temporary rise again but in very few cases was the maximum level maintained for more than 2 weeks.

The iron salts of several organic acids investigated were the acetate, albuminate, citrate, lactate, and tartrate. The literature records some discussion as to whether these salts are to be considered organic or inorganic. According to one authority (5), "It is to be emphasized that salts of iron which give the iron test

[2] Parke Davis and Company, Detroit.

directly are classed as inorganic iron, whatever their acid radicals
may be, and that true iron albuminate and iron peptonate are
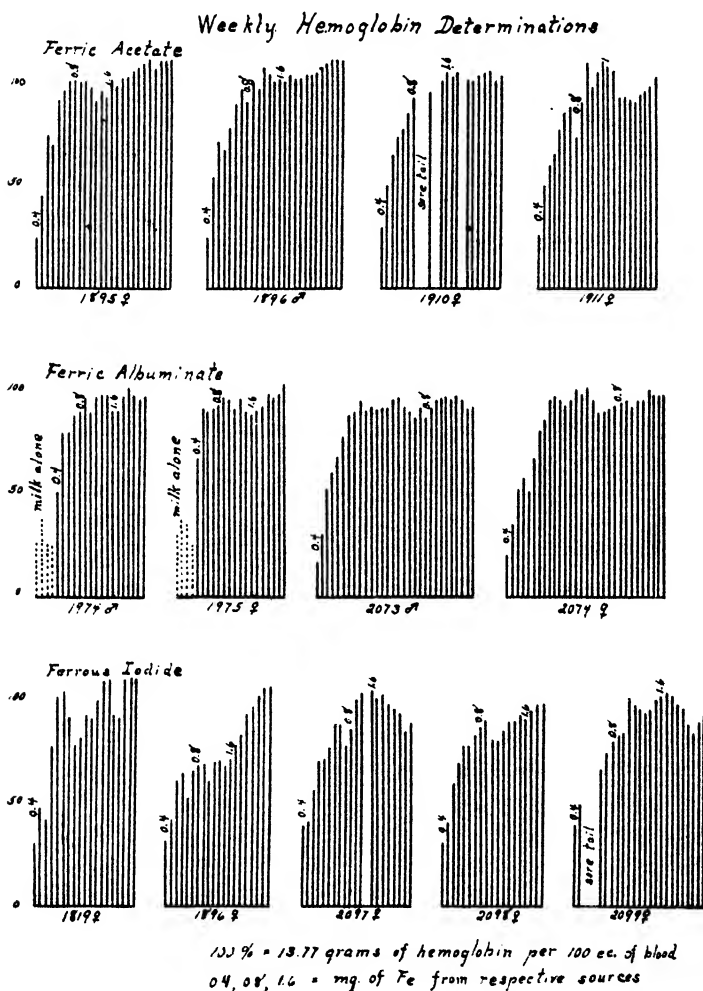

Weekly Hemoglobin Determinations

CHART III.

inorganic iron compounds." Whether there is general agreement
with this opinion or not is of little significance compared with the
fact that the compounds here investigated are not of the com-

plex or masked type found in animal and plant tissue and yet some of them are used for hemoglobin synthesis. Feeding tests with these salts have given widely varying results for which no satisfactory theory has been evolved, although differences in ionization, valence, and colloidal state have been suggested as possible causes of the differences.

Ferric acetate and ferric albuminate were tested on six and eight rats respectively and showed consistently good results when 0.4 mg. of Fe was fed with an average gain of 8.5 gm. of hemoglobin in 6 weeks on the acetate and of 8.2 gm. on the albuminate (Chart III). Furthermore, this rapid response was followed by a more gradual increase to an even higher level which was maintained throughout the experiment. As was mentioned in our previous paper (1) an increase in the quantity of iron supplement after a period of 6 or 8 weeks seems logical because of the increased demand of the larger but still rapidly growing animal. However, 0.4 mg. of Fe seemed to be sufficient for a considerable period of time as indicated by the records of Rats 2073♂ and 2074♀ (Chart III) where the improvement in the blood picture was continuous for 16 weeks before a slight reduction of the hemoglobin was noted and an increase to 0.8 mg. of Fe was made. Further evidence of the availability of the acetate and albuminate is offered by the prompt and rapid response made by rats which had failed to respond to 4 times the quantity of the less efficient iron salts; *viz.*, ferrous lactate, ferric potassium tartrate, and ferrum reductum (Chart IV) and peptonized ferric oxide (Chart I). It should be noted that in every case the recovery occurred on the minimum quantity of Fe from the better source.

Ferric citrate also stimulated a rapid response in four rats previously given lactate and tartrate (Chart IV). In our previous paper (1) ferric ammonium citrate was found to be an excellent source of iron but the question had been raised whether the somewhat less soluble ferric citrate would be equally available. Our evidence to date would indicate that either form of citrate is well utilized.

Ferrous lactate and ferric potassium tartrate, both soluble salts commonly used in remedies for anemia or chlorosis, failed to stimulate normal hemoglobin production in the thirteen animals

used. The average increase of 3.8 gm. of hemoglobin on the
lactate and 4.25 gm. on the tartrate in the first 6 weeks may in-
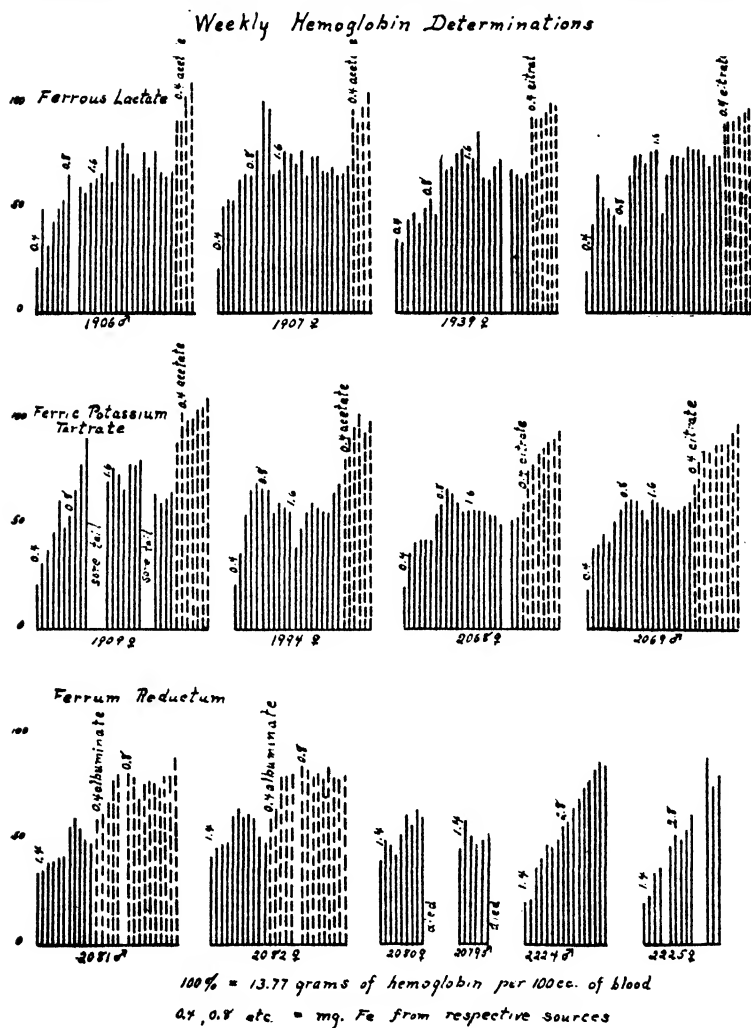

Weekly Hemoglobin Determinations

CHART IV.

dicate a small amount of utilization or a sparing action on body
iron, but it is quite evident from the charts that the increase to
1.6 mg. of Fe fed did not result in any further stimulation.   That

T. BL

Iron Compounds Used and Hemoglobin Response.

Iron compound.	Properties.	Minimum daily dose of iron (salt = 0.4 mg. Fe.)	No. of rats on experiment.	Average gain in body weight in 20 wks.		Average gain in hemoglobin in first 6 wks.	Maximum hemoglobin maintained for more than 2 wks. (in gm. per 100 cc. blood) on:		
				Males.	Females.		0.4 mg. Fe daily.	0.8 mg. Fe daily.	1.6 mg. Fe daily.
		mg.		*gm.*	*gm.*	*gm.*			
Ferric oxide, Fe_2O_3.	Red powder, insoluble in water, 70 per cent Fe.	0.57	12	200	126	3.5	4.7	7.2	11.0
Saccharated ferric oxide, $C_{12}H_{22}O_{11}$- $(Fe_2O_3)_2$ + $C_{12}H_{22}O_{11}Na_2O$.	Brown powder, soluble in water, 2.8 per cent Fe.	14.3	6	279	130	7.0	10.3	12.0	12.2
Peptonized ferric oxide, 5 per cent Fe_2O_3.	Brown powder, soluble in water, 3.5 per cent Fe.	11.43	5	206	139	6.9	9.6	13.1	11.9
Ferrous carbonate, $FeCO_3$.	Blaud mass, brown powder, insoluble in water, 25.6 per cent Fe.	1.56	12	200	140	2.0	8.5	11.4	
Saccharated ferrous carbonate, 15 per cent $FeCO_3$.	Greenish brown powder soluble in dilute HCl, 7.2 per cent Fe.	5.56	9	202	121	6.9	11.5	13.3	
Ferrous sulfate, $FeSO_4$·$7H_2O$.	Bluish green crystals, soluble in water, 20.1 per cent Fe.	2.0	6	100 (16 wks.)	130 (16 wks.)	2.9	4.8	8.5	

Ferric acetate, Fe₂(C₂H₃O₂)₆	Brownish red scales, soluble in water, 24 per cent Fe.	1.66	6	190	164	8.7	11.7	13.9	14.6
Ferric albuminate, 5 per cent Fe₂O₃.	Brown powder, soluble in water, 3.5 per cent Fe.	11.4	8	218	130	8.1	11.7	13.6	13.5
Ferrous iodide, FeI₂ + about 3H₂O.	Solution in ampules, 18 per cent Fe, stable in sucrose syrup.	2.21	9	228	135	6.2	10.1	12.1	13.7
Ferrous lactate, Fe(C₃H₅O₃)₂·3H₂O.	Greenish yellow powder, soluble in water, 19.4 per cent Fe.	2.06	5	217	165	3.9	6.6	9.8	10 1
Ferric potassium tartrate, Fe₂O₃K₂(C₄H₄O₆)₂.	Brownish red scales, soluble in water, 21 per cent Fe.	1.9	3	254	140	4.4	7.4	7.6	8.9
Ferrum reductum (reduced iron).	Gray powder, insoluble in water, 90 per cent Fe.	1.56 (1.4 mg. Fe.)	6	250	100	2.7		7 0 (1.4 mg. Fe.)	10.9 (2.8 mg. Fe.)

these rats were capable of building hemoglobin from an available source of iron is shown by their subsequent recovery on acetate and citrate.

Ferrum reductum or reduced iron (90 per cent Fe) is an insoluble gray powder which had to be incorporated into a starch paste for feeding.   For convenience and to insure a liberal consumption, this material was fed in larger quantities, 1.4 mg. of Fe at the beginning, increased later to 2.8 mg. of Fe daily.   A change of color to a reddish brown on the surface indicated some oxidation, so that the product fed was not pure reduced iron.   The hemoglobin response has been poor, averaging 2.5 gm. of hemoglobin in the first 6 weeks, with a decrease in some cases and a slow increase in two cases when the greater quantity was fed (Chart IV).   A striking rise in hemoglobin followed the change to albuminate after 12 and 14 weeks on reduced iron, showing that the rats still retained the capacity to recover.

Ferric cacodylate, which the physician would use hypodermically, was not found suitable for oral administration in rats as it caused diarrhea and loss of weight to such an extent that the experiment had to be discontinued after a few weeks.   However, no improvement in the blood picture was noted during that time.

Descriptions of the iron compounds used, the quantities fed, and response obtained are summarized in Table I.

<div style="text-align:center">DISCUSSION.</div>

Our previous publication (1) in which ferric chloride and ferric ammonium citrate were found to be far better utilized in blood building than ferric oxide and ferrous carbonate suggested that a possible explanation for this difference might be solubility.   After investigating a larger number of salts of varying solubility it appears that not all soluble salts are equally well utilized for hemoglobin synthesis, but we have never found an insoluble inorganic compound which was well utilized.   Complex organic compounds such as occur naturally in foods have not been investigated in this respect, but it is probable that these compounds are mostly insoluble in water but subject to solution or digestion by the digestive juices.

Hart et al. (6) question the purity of the salts utilized in our previous experiment but we feel that in the early stage of the

investigation, the use of a larger number of salts of standard quality may throw just as much light on an unsolved problem as more intensive work on just one or two compounds. Their conclusion that if soluble iron salts are purified their potency in respect to hemoglobin building is reduced in proportion to the purity, seems to be based upon their experience with ferrous sulfate only. In the absence of data on a pure iron salt their contentions would be more convincing if similar observations were made with several other soluble salts before such a sweeping conclusion were made. The differences noted between the various compounds reported in this paper need not necessarily be explained on the basis of impurities.

Furthermore, our results with ferric oxide, so largely used by Hart and his coworkers, have been so irregular that we question the accuracy of using it always in the basal diet. A few of our rats on ferric oxide made a fairly rapid recovery while others never responded or the response was greatly retarded. Hart's conclusions with regard to a factor which influences iron assimilation would be more conclusive if the materials tested were iron-free and had been tested on the rabbits without the presence of the iron oxide in the ration.

Our observations on the efficiency of ferrous sulfate *versus* ferric citrate for hemoglobin synthesis are quite in accord with findings recently reported by Simmonds, Becker, and McCollum (7), but we have no evidence to indicate that the presence or absence of vitamin E influences iron assimilation. Vitamin E has been supplied in abundance to our breeding colony and to the young rats until separated from the mothers. These young rats have been able to utilize some forms of iron and not others regardless of the possible storage of vitamin E and the amount already present in the milk. Furthermore, reproduction occurred quite normally when an abundance of vitamin E was supplied in spite of the marked iron deficiency. We have also observed xerophthalmia on some of our milk-fed rats, but it has not been confined to those receiving any specific source of iron nor to those without iron supplement. It occurred during the winter months and was attributed to lack of vitamin A, which theory was substantiated by prompt cures when cod liver oil was given.

The necesssity for great care in the choice of animals used and

in their previous diet history is illustrated by an incident which happened recently in our laboratory. Due to an oversight one female of our breeding colony had been receiving an available source of iron in its diet until mated. This rat's litter of young appeared just as anemic at weaning as those raised by the more anemic mothers but they showed spontaneous recovery on milk alone within a few weeks as well as more rapid recovery on certain iron salts than we had been able to demonstrate on any other litter.

A large number of rats has been fed on milk without iron supplement and we have never before observed this spontaneous recovery. Thirty rats from sixteen different litters have remained on the milk alone from 4 to 16 weeks and in no case has the blood picture improved. A temporary gain of 1 to 2 gm. of hemoglobin occurring in a few cases was always followed by a loss as great or greater than the gain. Two rats which remained on milk without iron supplement for 28 and 30 weeks, respectively, showed 2.9 and 3.4 gm. of hemoglobin per 100 cc. at the beginning and 3.0 and 3.1 gm. at the end of the experiment. Thus it is evident that the responses in hemoglobin which we have obtained must be due to the supplementary iron supplied rather than to a spontaneous recovery with age.

Contrary to the suggestion frequently offered that ferrous compounds are better utilized, it will be noted that the compounds which have shown the best availability in this series of experiments are all ferric, although some ferric salts have failed.

As a summary of this and previous work, iron salts may be grouped on the basis of the rapidity and height of hemoglobin response as follows:

Good.—Ferric acetate, ferric albuminate, ferric chloride, ferric citrate.

Fair.—Peptonized ferric oxide, saccharated ferric oxide, saccharated ferrous carbonate, ferrous iodide.

Poor.—Ferric oxide, ferrous carbonate, ferric potassium tartrate, ferrous lactate, ferrum reductum, ferrous sulfate.

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THE RELATION OF THE INORGANIC CONSTITUENTS OF A RATION TO THE PRODUCTION OF OPHTHALMIA IN RATS.

BY JAMES H. JONES.

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)

In 1922 McCollum, Simmonds, and Becker (1) described the production of an ophthalmia in rats on diets containing, what they thought to be, sufficient amounts of vitamin A. They believed that the ophthalmia was due to an improper balance of the inorganic constituents of the ration because their animals developed the ophthalmia if they fed their Salt Mixture 20, but not if they fed, instead, their Salt Mixture 185. In a second paper (2) these same investigators attempted to correlate the amount of vitamin B in the ration with the development of the sore eyes. In still more recent publications Simmonds, Becker, and McCollum (3) believe that they have shown a connection between the assimilation of iron and the reproductive factor, or vitamin E, both of which are associated in some unexplained manner with the eye trouble. They claim that the ferrous sulfate used in Salt Mixture 20 does not furnish the needed iron as well as the ferric citrate used in Salt Mixture 185, but if liberal amounts of vitamin E are present the ferrous iron is assimilated properly.

Mori (4) has shown by microscopical studies on the animals that the eye lesions are identical with those of the xerophthalmia resulting from a vitamin A deficiency.

Hopkins (5) has demonstrated that vitamin A is rather easily destroyed by oxidation. It is a well known fact that iron salts are good oxidative catalysts and especially at room or only slightly elevated temperatures. Karczag (6) has studied the effect of various inorganic salts on the speed of oxidative reactions. Of the salts investigated he found that the ferrous compounds had the

greatest accelerating effect and speaks of them as "oxidative catalysts par excellence." McCollum and associates used ferrous sulfate in the ration upon which the animals developed xerophthalmia and ferric citrate in the ration upon which the animals remained normal. It is highly probable that the different results obtained were due to a difference in the catalytic properties of the iron salts used.

The vitamin A in butter fat will remain potent almost indefinitely if the butter is kept in a solid mass and in a cool place. If, however, a small quantity of the fat is mixed with a dry ration air can mingle with it intimately. Under these conditions it is possible that vitamin A could be destroyed by atmospheric oxidation. Steenbock and associates (7) have pointed this out at various times, and they have adopted the procedure of incorporating the vitamin A-containing material (butter fat or cod liver oil) into the ration at intervals not greater than 1 week. In this way they have been able to prevent to a large degree the destruction of this vitamin after the ration has been prepared and before it is fed.

Experiments were started in 1925 for the purpose of comparing the ration used by McCollum and coworkers when it was prepared in quantities of 50 gm. per animal with the same ration when it was made up in quantities of 500 gm. per animal. The smaller quantity lasts about 5 days whereas the larger quantity lasts 6 or 7 weeks.

EXPERIMENTAL.

Albino rats which were raised on a standard stock ration were used as experimental animals. They were kept in cages with false screen bottoms. In most of the experiments the ration used was the same as that of McCollum and associates; viz., yeast 5, wheat gluten 12, gelatin 10, egg albumin 10, agar-agar 2, KH_2PO_4 1.7, butter fat 5, various amounts of salt mixtures, and dextrin to make 100. The butter was purchased in quantities of 10 pounds, melted, filtered, and then stored in the refrigerator, the temperature of which was just slightly above the freezing point. Except for the butter fat and the iron salt the rations were prepared in rather large quantities. The butter fat was incorporated into the ration as desired depending upon the individual
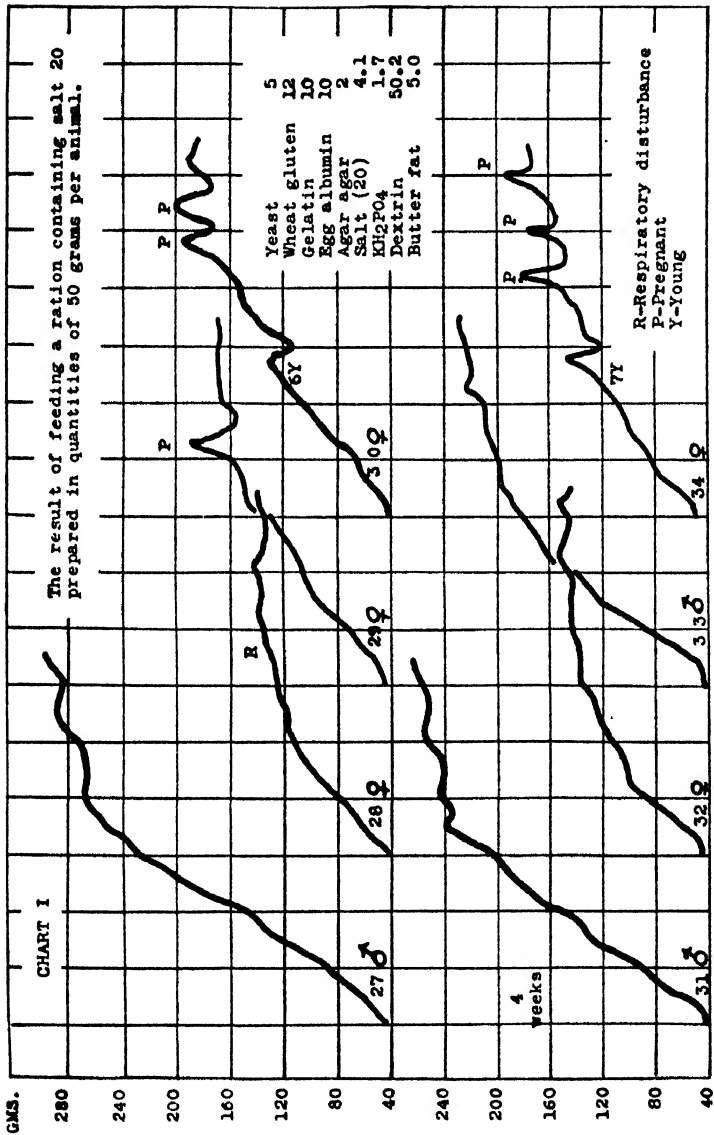
CHART I.

experiment. The iron salt was well mixed into the ration with the butter fat. This latter precaution was taken to prevent the ferrous salt from becoming oxidized to the ferric form before the butter fat was added. Feeding of the ration was begun immediately after adding the butter fat and iron salt.

The first experiment consisted of feeding eight rats on the ration which, according to McCollum, Simmonds, and Becker, regularly produced xerophthalmia. The butter fat was mixed into 50 gm. of ration per animal which lasted about 5 days. Chart I shows that the animals grew at nearly the normal rate for a period of 26 weeks, at the end of which time the experiment was discontinued. Two of the females reproduced, and there were several cases of pregnancy without parturition. None of the young lived more than 2 days. Rat 28 showed definite signs of respiratory disturbance.

This experiment was repeated twice, using a total of 24 animals. The last two experiments were continued for 16 instead of 26 weeks. The results were similar to those obtained in the first trial.

Identical experiments were conducted in which McCollum's Salt Mixture 185 replaced the KH_2PO_4 and Salt Mixture 20 of the previous ration. These animals grew no better, and externally they were in no way superior to those receiving Salt Mixture 20. If any difference could be detected it was in favor of the animals on the Salt Mixture 20 ration, as six of the twelve females receiving this ration reproduced and others showed pregnancies, whereas none of the females receiving Salt Mixture 185 gave birth to young although several of them became pregnant. *At no time did any of these animals show the least signs of an ophthalmia.*

When the rations were made up in the large quantities, 500 gm. per individual, the results obtained were very different. Three animals from the same litter (Chart II, Lot 35) which were so fed grew for a very short time; then failed with subsequent development of xerophthalmia. Three more animals from another litter (Chart II, Lot 31) reacted in a similar manner. Each animal developed xerophthalmia in less than 10 weeks with death following in a short time. To three more animals (Chart II, Lot 32) of the same litter as those of Lot 31 were given the same ration with the slight difference that it contained double the amount of ferrous

sulfate. These three animals developed xerophthalmia about 2 weeks sooner than their litter mates. This difference, however, is too slight to be of any importance.

Yeast	5
Wheat gluten	12
Gelatin	10
Egg albumin	10
Agar agar	2
Salt (20)	4.1
KH_2PO_4	1.7
Dextrin	50.2
Butter fat	5.0

CHART II. The result of feeding rations containing salt 20 prepared in quantities of 500 grams per animal

Same ration as above plus ferrous sulfate 0.2 per cent.

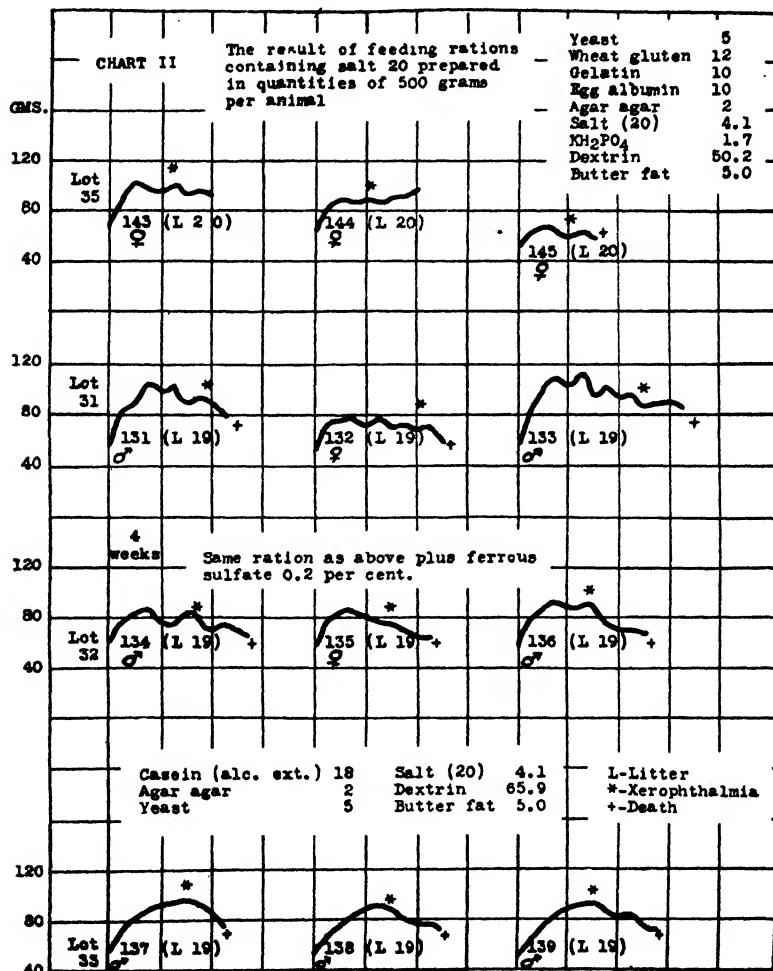|		
Casein (alc. ext.)	18
Agar agar	2
Yeast	5
Salt (20)	4.1
Dextrin	65.9
Butter fat	5.0

L-Litter
*-Xerophthalmia
+-Death

CHART II.

In still another experiment an entirely different ration, except for Salt Mixture 20 and butter fat, was fed. This ration was composed as follows: casein (alcohol-extracted) 18, agar-agar 2,

yeast 5, salt (No. 20) 4, butter fat 5, and dextrin 65.9. This diet was also prepared in quantities of 500 gm. per animal. The three animals (Chart II, Lot 33) in this experiment all developed xerophthalmia within 6 weeks and death followed at about the 10th week of the experiment.

In one of their papers McCollum *et al.* (2) state that if Salt Mixture 20 were reduced from a level of 4.1 per cent to 2 per cent of the ration the time elapsed before the onset of xerophthalmia was very much increased. If the trouble lies with the ferrous
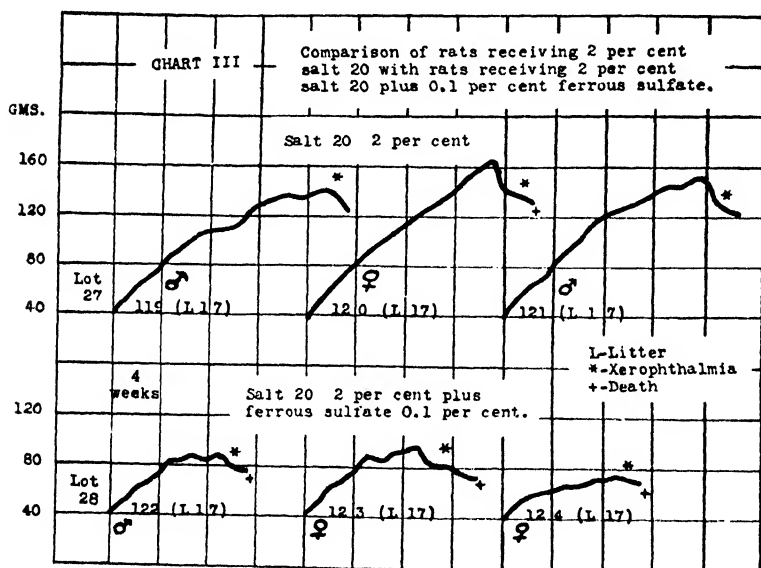
CHART III.

sulfate alone the results obtained should depend upon the amount of this ingredient in the ration, and not upon the amount of other inorganic constituents present. Therefore, if sufficient ferrous sulfate were added to the ration containing 2 per cent of the salt mixture to equal the ferrous sulfate content of the ration containing 4.1 per cent of the salt mixture, the results should be the same as those obtained when the whole salt mixture is fed at the higher level. Three animals were fed a ration, containing 2 per cent of Salt Mixture 20. The ration was prepared in an amount equiv-

alent to 500 gm. per animal. The animals grew almost normally for several weeks and did not develop xerophthalmia until about the 19th week. Three other animals from the same litter were given the same ration with double the amount of ferrous sulfate, or an additional 0.1 per cent. These animals failed much sooner than their litter mates with the onset of xerophthalmia in about one-half the time. Chart III gives the result of·this experiment which indicates that, other conditions being similar, the time elapsing before the onset of xerophthalmia is dependent on the amount of ferrous sulfate in the ration.

These data show that the development of xerophthalmia in rats when fed the ration used by McCollum, Simmonds, and Becker is not due to a hitherto unknown type of nutritive disturbance. Likewise there has not been demonstrated a relation of iron assimilation to any of the vitamins. Instead, the condition is probably one of simple vitamin A deficiency caused by the oxidative destruction of the vitamin in the ration. The rate of oxidation is increased by the catalytic action of ferrous sulfate.

Data which as yet are too meager for presentation indicate that it is not only a question of the type of iron salt that is used but also the amount. When McCollum and associates fed their Salt Mixture 20 at a level of 4.1 per cent they were feeding about twice as much iron as they were when they fed their Salt Mixture 185 at a level of 3.7 per cent. Data concerning this phase of the question will be presented in a later publication.

SUMMARY.

1. The ration containing 5 per cent of butter fat which McCollum, Simmonds, and Becker claim regularly leads to the production of xerophthalmia in rats has been investigated.

2. If the butter fat is incorporated into this ration at intervals of about 5 days the animals grow normally and do not develop sore eyes.

3. If the butter fat is added to the same ration in quantities sufficient to last 6 or 7 weeks xerophthalmia develops in the course of 10 weeks or less with subsequent loss of weight and death of the animals.

4. If the butter fat is incorporated at the less frequent intervals

the time elapsing before the development of xerophthalmia depends upon the amount of ferrous sulfate in the ration.

5. It is suggested that the failure of the animals under these conditions is due to an oxidative destruction of vitamin A, the speed of oxidation being increased by the catalytic action of the ferrous sulfate.

In the June 1927 number of the *Proceedings of the Society for Experimental Biology and Medicine,* Simmonds, Becker, and McCollum (8) have published a short note indicating that they have now recognized that their so called salt ophthalmia can be cured when the ration containing ferrous sulfate is made up daily. The data which comprise the present paper were presented in detail before The Physiological Society of Philadelphia at the April, 1927, meeting and an abstract has already appeared elsewhere (9).

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OBSERVATIONS ON THE ABSENCE OF HYPOGLYCEMIA AFTER THE INTRAPERITONEAL INJECTION OF INSULIN IN WELL FED RABBITS.

By F. MEREDITH HOSKINS and FRANKLIN F. SNYDER.

(From the Department of Anatomy, the University of Rochester School of Medicine and Dentistry, Rochester, New York.)

INTRODUCTION.

The effect of insulin on the blood sugar following intraperitoneal injection has received little attention. It is clear, however, from anatomical considerations that the path of absorption of insulin introduced into the peritoneal cavity parallels more closely the normal anatomical pathway by which insulin reaches the circulation from its site of origin in the pancreas than is the case when it is injected by other routes—subcutaneous, intramuscular, or intravenous.

This is evident when one bears in mind that the blood which bathes the islets of Langerhans is collected by tributaries of the portal vein and carried into the liver, where it is again spread out in an extensive network of porous capillaries or sinusoidal spaces, finally draining through the hepatic veins into the vena cava. Likewise, the path of absorption of soluble material, such as phenolsulfonephthalein, introduced into the peritoneal cavity is by way of the veins to a large extent, rather than through lymphatics (Dandy and Rowntree, 1913). On the other hand, by injection of insulin subcutaneously, intramuscularly, or intravenously, there is a departure from the normal physiological conditions in proportion to the journey which the insulin must make through other organs before it can reach the portal vein and liver.

It is a striking fact, and not without significance as our experiments show, that normally the internal secretion of the pancreas always comes into close relationship with the liver cell before it is cast into the general circulation.

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Previous observations on the intraperitoneal injection of insulin in the rabbit are not numerous.   Choay (1926, p. 216) cites the observation of Bénard and Choay that a rabbit which had been fasted 24 hours went into convulsions after injection of insulin intraperitoneally.

In recent observations of Barbour, Macleod, *et al.* (1927), insulin given intraperitoneally in rats is found to be followed by hypoglycemia under the conditions of the experiments.

It is evident from the literature that while hypoglycemia has been noted after insulin given intraperitoneally in animals which have fasted, the action of insulin in well fed rabbits after intraperitoneal injection seems to have been untried.   In view of the limited information which we were able to gather by reading and the interesting possibilities suggested by a few casual observations on rabbits, we have attempted to observe the effect on the blood sugar of insulin given intraperitoneally in two groups of rabbits, taken at opposite phases in carbohydrate metabolism.

Method and Material.

The general plan was to follow the blood sugar after the intraperitoneal injection of insulin in one group of rabbits which was well fed in contrast to another group which had fasted 24 hours or longer.

In the first group the animals were taken directly from cages which contained at all times an abundant supply of oats, alfalfa hay, water, and cabbage or carrots.   They were weighed and a blood sample of about 2 cc. was taken from an ear vein.   Insulin was then injected with a 1 cc. tuberculin syringe into the peritoneal cavity near the midline, about half way between the umbilicus and symphysis pubis.   A dose of about 3 units per kilo of body weight was usually given, which was about twice the calculated convulsive dose.   The animal was then left in a large box in the laboratory without food.   About 2 hours after the injection of insulin another blood sample was taken from the ear vein. Twenty-eight rabbits were used in this group in a total of thirty-one experiments (Table I).

In the second group, the rabbits were removed from the cages

TABLE I.

Intraperitoneal Insulin, Rabbits Not Fasted.

Rabbit No.	Sex.	Weight.	Units of insulin injected intraperitoneally.	Time of insulin injection.	Time of taking blood samples.	Blood sugar.	Date.
		kg.				*mg. per 100 cc.*	
2	♂	2.3	5	8.23	8.17	131	Apr. 21
					9.15	123	
					10.30	115	
4	♂	3.2	6	8.55	8.55	131	May 7
					10.55	123	
6	♂	3.3	10	9.13	9.08	112	May 7
					11.10	123	
7	♂	3.6	10	9.05	9.00	133	May 7
					11.05	123	
12	♂	2.7	8	3.35	3.30	120	May 12
					5.35	106	
					7.35	114	
14	♂	3.0	10	4.05	4 00	106	May 11
					6.05	103	
					8.05	113	
15	♂	3.0	9	3.46	3.41	105	
					5.46	102	
					7.46	98	
23	♀	4.1	12	2.38	2.35	110	
					4.35	110	
29	♀	2.4	7	1.43	1.40	136	
					3.45	116	
29	♀	2.8	8.5	3.15	3.12	143	
					5.10	135	
30	♀	2.2	6.5	1.48	1.45	119	
					3.50	106	
33	♂	3.4	10	1.30	1.25	115	May 14
					3.25	108	
33	♂	3.1	10	2.58	2.55	119	June 5
					4.55	125	
36	♀	2.0	6	1.45	1.43	105	May 14
					3.45	100	
38	♂	3.0	9	2.21	2.19	125	June 4
					4.21	135	
39	♂	2.6	8	2.31	2.30	120	June 4
					4.32	126	
40	♂	2.5	8	2.43	2.40	130	June 4
					4 42	126	

TABLE I—*Concluded.*

Rabbit No.	Sex.	Weight.	Units of insulin injected intraperitoneally.	Time of insulin injection.	Time of taking blood samples.	Blood sugar.	Date.
		kg.				*mg. per 100 cc.*	
43	♂	3.0	9	2.43	2.40	130	
					4.43	121	
1	♂	1.9	3.5	1.15	1.00	125	
					3.15	90	
					4.45	112	
6	♂	3.2	10	1.35	1.33	116	
					3.32	58	
7	♂	3.4	10	1.40	1.38	118	
					3.36	55	
13	♂	3.2	10	3.55	3.50	127	
					5.55	50	
					7.57	76	
16	♂	3.0	10	4.12	4.10	113	
					6.13	51	
					8.12	89	
21	♀	2.7	10	2.26	2.23	112	
					4.23	68	
22	♀	3.5	11	2.32	2.30	117	
					4.30	55	
24	♀	3.9	11	2.45	2.42	112	
					4.42	73	
31	♀	2.1	6.5	1.55	1.55	110	
					3.57	43	
32	♂	2.0	6	2.01	1.57	112	
					4.05	89	
37	♀	2.3	8	1.50	1.47	118	
					3.05	45	
41	♀	2.7	8	2.38	2.37	134	
					4.38	55	
42	♂	3.2	10	2.33	2.30	135	
					4.33	62	

which contained an abundant food supply into empty ones, and were allowed only water for a period of about 1, 2, or 3 days before the experiment. This group was injected in a similar manner to the first lot, with the exception that a dose of insulin only half as large was usually used; that is, about 1½ units per kilo of body weight. Eighteen rabbits were used in a total of twenty-one ex-

TABLE II.
Intraperitoneal Insulin, Fasted Rabbits.

Rabbit No.	Sex.	Duration of fasting.	Weight.	Units of insulin injected.	Time of insulin injection.	Time of taking blood samples.	Blood sugar.	Date.
		hrs.	*kg.*				*mg. per 100 cc.*	
2	♂	24	2.1	7	2.11	2.09	102	May 12
						4.09	40	
3	♂	24	2.5	20	7.38	7.30	107	
						9.50	48	
4	♂	24	3.0	5	9.45	9.35	100	Apr. 26
						11.35	35	
4	♂	24	3.2	6	4.20	4.18	105	May 9
						6.18	53	
6	♂	24	3.2	10	4.12	4.10	92	May 9
						6.12	87	
7	♂	24	3.2	10	4.04	4.00	116	May 9
						6.04	48	
20	♀	24	2.8	8	2.15	2.12	106	
						4.12	52	
12	♂	48	2.7	4	10.23	10.20	112	June 8
						12.23	55	
33	♂	48	3.0	11	10.12	10.05	112	June 8
						12.12	49	
33	♂	48	3.4	7	7.19	7.12	120	May 17
						9.12	60	
36	♀	48	2.0	4	7.25	7.22	112	May 17
						9.25	50	
38	♂	48	2.7	4	2.31	2.28	101	June 7
						4.31	37	
39	♀	48	2.3	3.5	2.35	2.20	104	June 7
						4.25	56	
40	♂	48	2.2	3.5	2.40	2.35	112	June 7
						4.42	41	
45	♀	48	2.5	3.5	10.15	10.14	124	
						12.15	42	
17	♀	62	2.6	8	1.55	1.50	130	
						4.00	50	
18	♂	62	3.4	6	2.05	2.00	121	
						4.02	40	
12	♂	78	2.5	4	9.17	9.15	112	May 18
						11.17	58	
13	♂	78	2.9	5	9.00	8.55	108	May 18
						11.00	42	
14	♂	78	2.6	4.5	9.11	9.08	102	May 18
						11.11	48	
18	♂	78	3.5	6	9.06	9.02	120	
						11.06	37	

periments with this group. Among the eighteen rabbits upon which observations were made after fasting, there were eleven which were also observed previously when well fed. In Tables I and II the complete record of every animal is listed.

Stock rabbits were used, which were not especially selected for the experiments. No pregnant does were included.

Lilly's commercial insulin containing 10 units per cc. was employed throughout. The activity of every lot of 10 cc. was controlled by subcutaneous administration or by intraperitoneal injection in fasted rabbits.

Blood sugar determinations were made according to the method of Folin and Wu (1920).

Observations.

After intraperitoneal injection, the influence of insulin on carbohydrate metabolism, as indicated by the blood sugar concentration, was in marked contrast in two groups of rabbits. The first group consisted of well fed animals, while the second group had been deprived of food 24 hours or longer.

In observations on well fed rabbits after the injection of insulin intraperitoneally, we have noted in the majority of cases no marked lowering of the blood sugar. In a total of thirty-one experiments carried out on twenty-eight rabbits, the blood sugar remained at nearly a constant level in eighteen cases, while in thirteen cases there was a fall which exceeded 20 mg. of sugar per 100 cc. of blood. The dose of insulin used in this group of animals was usually 3 units per kilo of body weight. Observations on the blood sugar were based on two samples of blood, one taken just before the insulin injection and the second obtained 2 hours later. In six instances, however, a third sample was taken 4 hours after injection.

In the group of fasted rabbits, on the other hand, the blood sugar was strikingly affected by the intraperitoneal injection of insulin. The sugar concentration was lowered very markedly. In twenty-one experiments on eighteen animals there was a fall in blood sugar averaging 60 mg. per 100 cc. of blood. In a single case, no lowering of the blood sugar was noted. The dose of insulin injected in this group of animals is about 1½ units per kilo of body weight, or only half of the amount which was used in the group of well fed rabbits.

These observations, made upon two separate groups of animals, have been checked by another series made by studying individual rabbits at the two extreme phases of carbohydrate supply; *i.e.*, in the well fed condition in contrast to the fasting stage. Twelve experiments were performed with rabbits in which insulin was first administered in the well fed condition. In all of these the blood sugar remained at a nearly constant level. After completion of the first observations, the animals were well fed for at least 24 hours and thereafter fasted 24 hours or longer. Administration of insulin in the fasting stage led in all cases but one to a marked lowering of the blood sugar. As noted in Tables I and II, the animal, when well fed, received about twice the dose of insulin which was injected after fasting; and in many instances the same bottle of insulin was used in both observations on the same animal.

DISCUSSION.

From the foregoing observations it is evident that the absence of hypoglycemia in the well fed rabbit after the injection of insulin by the intraperitoneal route stands alone and is in striking contrast to the effect on the blood sugar after injection by other routes—subcutaneous, intramuscular, and intravenous. The observations of many investigators agree regarding the marked hypoglycemia which occurs in well fed rabbits after the injection of insulin subcutaneously, intramuscularly, or intravenously (McCormick, Macleod, *et al.*, 1923). Therefore, the failure of the blood sugar to fall after intraperitoneal injection would seem to be definitely associated with the anatomical pathway followed by the insulin.

In tracing the course followed by insulin after injection into the peritoneal cavity, the question arises as to whether insulin actually reaches the blood stream or whether it is somehow altered or destroyed by the contents of the peritoneal cavity or the mesothelial lining. It is obvious that absence of hypoglycemia would result, if the insulin were destroyed. That active insulin can reach the blood stream by the intraperitoneal route without destruction is shown, however, by the marked lowering of the blood sugar in the fasted rabbit. Furthermore, the amount of insulin injected in the well fed rabbit is twice as great as that given to the fasted animal, in which hypoglycemia consistently results.

In addition to the evidence that insulin can reach the blood stream from the peritoneal cavity, there are also observations which indicate that this path must lie chiefly through the veins rather than by way of the lymphatics which drain into the thoracic duct.   Thus, Dandy and Rowntree found that in two dogs in which phenolsulfonephthalein was introduced intraperitoneally, there was an output of more than 50 per cent of the dye in the urine within an hour after injection, while during the same time in the lymph escaping from a cannula in the thoracic duct, there was only a trace of dye, less than 0.1 per cent.   The path of venous drainage of the peritoneal cavity must lie through the portal vein and liver to a considerable extent, although it is difficult to determine the relative amount of absorption from the peritoneal cavity which may occur by way of veins which do not empty into the portal system, since direct observations appear to be lacking.   In our experiments, in so far as insulin enters the blood stream by way of the tributaries of the portal vein, it must first pass through the liver on the way to the general circulation.   It is evident that the passage of insulin through the liver before the muscles and other peripheral tissues are reached stands forth as a striking difference between injection by the intraperitoneal route in contrast with other routes—subcutaneous, intramuscular, or intravenous.

In connection with the observations which point to the liver as the site of operation of the reaction which so modifies the effect of insulin on the blood sugar that hypoglycemia is prevented, it has also been noted that this effect on insulin action is dependent upon whether the rabbit is well fed or fasted.   It becomes a question of what substance, occurring in the liver of a well fed rabbit and vanishing with fasting, can react with insulin to prevent hypoglycemia.   One might infer that it is glycogen, in view of the many observations in the literature which show that the glycogen content of the liver is very greatly reduced in a rabbit which has fasted 24 hours or longer (Macleod, 1926).

From the foregoing considerations a clue is suggested toward the solution of the persistent problem which is presented by the occurrence of hypoglycemia in well fed rabbits with large stores of glycogen in the liver and muscles, when insulin is given intravenously or subcutaneously.   Obviously these routes of adminis-

tration may be inadequate to reveal the complete mechanism of insulin action on the blood sugar, since insulin reaches the muscles and other tissues before it can enter the liver with its huge glycogen store.   In considering the mechanism of hypoglycemia, it is necessary to take into account both the route of administration of the insulin and the glycogen stores of the animal.   Thus, in the fasted rabbit injected with insulin by the intraperitoneal route, the occurrence of hypoglycemia is linked with a real glycogen deficit in the liver.   On the other hand, in the well fed rabbit in which insulin is administered by other routes—subcutaneous or intravenous—the occurrence of hypoglycemia seems to indicate that glycogen, though actually present in the liver, may remain unavailable to such an extent that marked lowering of the blood sugar ensues.

The absence of hypoglycemia after the intraperitoneal injection of insulin points to the presence of a compensatory factor in the liver which tends to maintain the blood sugar at a constant level. Since this factor vanishes in the fasting rabbit, it may well be related to glycogen.

CONCLUSION.

In well fed rabbits, insulin injected intraperitoneally is in most cases not followed by a lowering of the blood sugar such as occurs in fasting animals similarly injected.

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THE SEPARATION OF A SUBSTANCE FROM OILS WHICH INHIBITS THE DESTRUCTION OF VITAMIN A BY FERROUS SULFATE.

By HOWARD W. ESTILL and E. V. McCOLLUM.

(From the Department of Chemical Hygiene, School of Hygiene and Public Health, the Johns Hopkins University, Baltimore.)

In a previous paper (1) from this laboratory it was reported that the ophthalmia induced in rats by feeding ferrous sulfate in the food mixture can be prevented or cured by the subsequent addition of wheat germ oil to the ferrous sulfate-containing ration. In the case of a fat which contains vitamin A, it has been shown that ferrous sulfate causes the destruction of the vitamin, and this takes place rapidly with butter fat but more slowly with cod liver oil (2). This destruction, or possibly, inactivation, is inhibited by the inclusion of a small amount of wheat germ oil in the diet.

In investigating other possible avenues of attack than those used heretofore in the isolation of fat-soluble vitamins, we have separated a most interesting substance from cod liver oil in the form of its lithium chloride derivative; this substance, or active fraction, is very effective in inhibiting the destruction of vitamin A by ferrous sulfate.

Zwikker (3) reported that when lithium chloride is dispersed in a fat by the addition to the latter of a solution of the anhydrous salt in pyridine the cholesterol is precipitated practically quantitatively in the form of a crystalline compound consisting of 1 mol each of cholesterol and lithium chloride. It was recommended that the system be diluted with petroleum ether to facilitate filtering. We have not found the reagent so effective for the quantitative removal of cholesterol from a fat or oil as Zwikker believed it to be because of the slight solubility of the lithium chloride compound in the oil-pyridine system. The compound is easily prepared from anhydrous lithium chloride and cholesterol dissolved in dry pyridine.

It is well known that the more common alcohols form double compounds with a number of salts. The best known of these are the complexes formed from an alcohol with LiCl, $MgCl_2$, $CaCl_2$, BaO, Al_2Cl_6, $SnCl_2$, $SbCl_5$, $CuSO_4$, $Mg(NO_3)_2$, and $PtCl_4$. The lithium chloride derivative of the substance described in this paper is typical of several derivatives which can be produced from the higher alcohols and sterols accompanying fats and oils by employing certain of the salts listed above under appropriate conditions in non-aqueous systems; the usefulness of this type of reaction in the isolation of the fat-soluble vitamins is under investigation in this laboratory.

Since the studies of Takahashi (4) on vitamin A, and those of Steenbock (5), Hess (6), and Hess and Windaus (7) on the development of antirachitic properties in cholesterol or ergosterol by irradiation with ultra-violet light appear to indicate that both vitamins A and D are related to the sterols (which contain hydroxyl groups), it was thought possible that one or more of the fat-soluble vitamins could be separated from the fats in which they are dissolved by forming derivatives with those salts which combine with alcohols in non-aqueous systems. We report at this time the separation from cod liver oil of a lithium chloride complex which induces in rats a recovery from the "salt ophthalmia" due to feeding ferrous sulfate in a manner comparable to the inclusion of wheat germ oil in the ration. The method of preparing the substance is as follows: To 200 gm. of cod liver oil diluted with 216 cc. of petroleum ether were added quickly and with constant rotating 3 gm. of anhydrous lithium chloride dissolved in 38 cc. of anhydrous pyridine. Because pyridine is a rather strong base, and since its effect on the fat-soluble vitamins is entirely unknown, it seemed best to exclude air from the reaction mixture by means of carbon dioxide; this also prevented any oxidation of vitamin A by contact with air. The latter was excluded by saturating the cod liver oil-petroleum ether mixture with carbon dioxide before adding the lithium chloride-pyridine reagent. At the time of mixing, the temperature of the diluted cod liver oil was 11° and that of the lithium chloride solution, 20°. As soon as the solutions were mixed, a white precipitate formed in rather large amount; most of this was doubtless lithium chloride. The mixture was rotated vigorously for $\frac{1}{2}$ hour in a stoppered

1 liter Erlenmeyer flask; during this time carbon dioxide from a cylinder was introduced frequently and any ether lost was replaced. Toward the end of the reaction period one noticed that that part of the precipitate which had settled out most readily had assumed a very light pink color; the supernatant liquid was very turbid. After centrifuging for about 30 minutes at a moderate speed, the virtually clear solution was decanted as much as possible, and the precipitate (some of it pasty, some granular) transferred quickly to a No. 50 Whatman filter, and filtered as dry as possible under diminished pressure; the very deliquescent precipitate was protected by means of pure dry carbon dioxide. Petroleum ether was used to wash the precipitate free from cod liver oil and pyridine; the precipitate was then removed to a 100 cc. centrifuge tube (filled with dry CO_2) and 50 cc. of petroleum ether added at once. After stirring the precipitate thoroughly, one noticed that some of it in the form of small pellets had risen to the surface of the liquid. Below this part of the precipitate the solution was very turbid, while a rather coarsely granular light pink colored precipitate was noted at the bottom of the tube. That portion of the precipitate which floated on the surface of the petroleum ether consisted entirely of imperfectly spherical granular pellets (diameter about 1 mm.) which resembled particles of sago. It was impossible to obtain a clear idea of the physical nature of the individual pellets of the precipitate of low specific gravity, since their appearance under the microscope varied greatly with the method of illumination; but one gained the impression that essentially they consisted of thin shells enclosing irregularly formed masses. As the precipitate aggregated at the surface of the liquid it had a faint pink cast; this precipitate will be referred to as "the precipitate of low specific gravity." There was nothing particularly characteristic about that portion of the initial precipitate which rendered the solution turbid; it was designated "the finely divided precipitate." The coarsely granular portion of the initial precipitate was definitely cystalline; it was called "the coarse precipitate."

It is believed that a fairly clean cut separation of the precipitate of low specific gravity was effected; certainly none of the coarse precipitate separated with it, and the small amount of the fine precipitate carried with it was easily washed out with petroleum

ether.   Much less satisfactory was the separation of the fine and coarse precipitates; but it was thought that it was sufficiently exact to enable one to decide which precipitate, if either, had carried down one or more of the fat-soluble vitamins.

The precipitate of low specific gravity was removed with a small flexible spatula to a small glass evaporating dish (or "shell") containing petroleum ether in contact with a gentle stream of pure dry carbon dioxide.   After removing as much of this precipitate as possible, the precipitate in the centrifuge tube was again stirred up gently but thoroughly, and more of the precipitate of low specific gravity removed to the glass shell.   This operation was repeated until no more of the precipitate floated on the surface of the petroleum ether.

The exposure of a small amount of this precipitate to the air showed that it was extremely hygroscopic.   It was therefore carefully protected against moisture with the aid of petroleum ether and dry carbon dioxide.   It was necessary to use carbon dioxide, for some of the precipitate rose to the surface of the petroleum ether.   The precipitate was then washed with petroleum ether to remove the remaining cod liver oil and pyridine, and the latter was tested for by shaking a given portion of the filtrate with water and adding an excess of a saturated solution of mercuric chloride to the aqueous layer (in the presence of pyridine a white crystalline precipitate is formed).   The precipitate must have been free from cod liver oil for from 500 to 1000 cc. of petroleum ether were necessary to remove the pyridine.

In order to remove lithium chloride, 4 cc. of water were added to the precipitate, which dissolved immediately.   Above the aqueous layer was a narrow, very light yellow colored oily layer.   The solution was treated at once with 10 cc. of ethyl ether, then transferred to a separatory funnel, and the glass shell rinsed with 5 cc. of ether.   The aqueous layer was washed with three 10 cc. portions of ethyl ether, and the washings added to the main portion. The colorless ethereal solution was evaporated on dextrin in a current of air and tested for the relief of salt ophthalmia.

In order to investigate the active substance or fraction, the ether was removed from the ether extract of the hydrolyzed precipitate of low specific gravity with the aid of dry carbon dioxide, and at a temperature not to exceed 40°.   A small amount of a pale

yellow pungent oil remained. This concentrate gave an entirely negative Liebermann-Burchard reaction, sulfuric acid reaction, and Drummond AsCl₃ reaction (7) for vitamin A. Chemical studies, including other color reactions, are now in progress.

The ether extracts of the three hydrolyzed precipitates, that is, the precipitate of low specific gravity, the fine precipitate, and the coarse precipitate, were combined and tested for vitamin A; other ether extracts similarly prepared from the three precipitates were combined and tested for vitamin D. In each case the animal tests were negative. The treatment of both the fine and coarse precipitates was substantially the same as described above for the precipitate of low specific gravity. It may be mentioned that ether extracts of the separately hydrolyzed fine and coarse precipitates were found to be entirely negative when tested individually for vitamins A and D and for the relief of salt ophthalmia.

From the manner in which the lithium chloride complex separates from the accompanying material it would seem probable that the preparation consists largely or wholly of an individual organic substance. We have employed this lithium chloride-free material (lithium chloride being toxic to rats) in the type of experiment described in a previous paper (1). In most instances the entire yield of the organic portion of the precipitate of low specific gravity obtained from 200 gm. of cod liver oil was thoroughly mixed with 40 gm. of dextrin; this dextrin was then used to make up to 200 gm. of ration.

An attempt to remove the same substance from wheat germ oil resulted in the separation of the active principle with lithium chloride; but in contrast to the case of cod liver oil, a clean cut physical separation from accompanying substances was not effected. We are pursuing further the perfection of a method of separation of the components of the mixture obtained from wheat germ oil.

From the known property of lithium chloride to form compounds with alcohols, it is of interest that a very small amount of a substance with the properties described can be separated by combining it with lithium chloride from fats containing vitamin E (wheat germ oil and cod liver oil). Careful tests of the material composing the organic portion of the lithium chloride complex for vitamins A and D were entirely negative. We have experienced much

difficulty in securing female rats of proven sterility by using the original diet of Evans (8) for inducing resorptions in rats. For this reason we have not yet been able to make conclusive tests on the material described for vitamin E with female rats, but such results as have been obtained and certain observations on male rats which have been kept on diets which induce sterility in males seem to indicate that the substance which we have separated by the method described may be vitamin E. Further studies of this interesting material are being conducted.

CONCLUSIONS.

1. The destruction of vitamin A by ferrous sulfate is inhibited by a substance separated from certain oils by means of lithium chloride dissolved in pyridine; this substance may be vitamin E.

2. The alcohol-salt addition product type of reaction promises to be useful in vitamin isolation.

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THE CROSSED DISMUTATION BETWEEN ALDEHYDES AND KETONES.

I. BENZALDEHYDE AND MENTHONE, AND THEIR SIGNIFICANCE FOR THE MENTHONE-MENTHOL RELATIONS IN MENTHA PIPERITA L.

BY SAMUEL M. GORDON.*

(From the Laboratory of Plant Chemistry, University of Wisconsin, Madison.)

In a previous report (1) the author has described the probable course of the genesis of isoamyl alcohol in *Mentha piperita* L. Isovaleric aldehyde is postulated as the intermediate product, the alcohol being derived by a process of autoreduction with the simultaneous oxidation of a second molecule of aldehyde to isovaleric acid, within the plant. Such reactions as were postulated are assumed in a number of phytochemical reactions, wherein the peculiar relationship of aldehyde, alcohol, and acid exist side by side, either free or as esters. A tabulation of these compounds as they occur in plant products argues for the possibility of such reactions within the plant, as has already been pointed out (2). Such a relation immediately calls to mind the well known Cannizzaro reaction, which has, in both the original and modified form, served as a model for the biological explanation of such phenomena in the plant.

It had previously been shown by others that, in the decomposition of carbohydrates within the cell, intermediate products, which are brought into reaction by a "dismutation" process, play an important part. The general scheme of sugar decomposition proceeds according to the assumption of a series of such dismutation processes (3). Such dismutation processes have also been shown to take place in acetic acid fermentation and for a number of aldehydes (4).

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* National Research Council Fellow in the Biological Sciences.

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The reaction for pairs of aldehydes, either similar or dissimilar, is well known through the work of Lieben, his associates, Tischtschenko, and more recently of Adkins and his students. The biochemical applications of the reaction have particularly been studied by Neuberg, Nord, Wieland, and others. Neuberg (5) and Endoh (6) have furnished excellent reviews of the biological aspects.

The reaction has been studied extensively for simple and mixed pairs of aldehydes, but the reaction between an aldehyde, on the one hand, and a ketone on the other, has been less completely investigated. Neuberg (5) was the first to demonstrate a crossed dismutation between an aldehyde and a ketone. By means of aluminum ethylate, he obtained the benzoic acid ester of methyl-n-propyl alcohol from benzaldehyde and methyl-n-propyl ketone. He also pointed out the importance of the reaction in phytochemical reduction processes. A tabulation of ketones and their related secondary alcohols occurring side by side in plant products seems to bear out his statement.

In this paper is recorded a crossed dismutation between benzaldehyde and menthone by means of aluminum ethylate. In a non-aqueous medium the benzoic acid ester of menthol was obtained, which on hydrolysis yielded menthol and benzoic acid. The reaction illustrates the further general applicability of the Cannizzaro reaction, menthone belonging to the so called hydroaromatic series, no member of which up to now has been known to undergo this reaction.

The yields of the ester are, however, far from satisfactory, and are much less than when 2 molecules of aldehyde react. The reason for this may be due to thermodynamic causes; and further, we are led to expect that the reaction between an aldehyde and a ketone will not proceed smoothly, because of the possibility of the many side reactions. In fact, the larger part of the benzaldehyde is converted to benzyl benzoate, and much of the ketone is recovered unchanged. Starting with gram molecular amounts of menthone and benzaldehyde, we obtained only 33 gm. of menthyl benzoate. This amount corresponds to 12.6 per cent.

The benzoate obtained, however, was an isomeric form of the menthol ester, viz., l-neomenthyl benzoate, which is in agreement with an observation of Zeitschel and Schmidt (7).

The immediate purpose for the study of this reaction was to throw some light on the formation of menthol in certain species of *Mentha*. It has been suggested by Pickard and Littlebury (8) in 1912 that menthone may be the precursor of menthol in the peppermint plant, and that the latter is formed by a reduction process, without offering a mechanism by which the reduction takes place. Several years later Kleber (9) found that oil from younger plants contained a larger amount of menthone than did fully developed plants. Hence, taken in conjunction with the work of Pickard and the analytical results of Kleber, the reaction suggests that menthol may indeed find its precursor in menthone and may be formed from it by a simultaneous oxidation-reduction process; *viz.*,
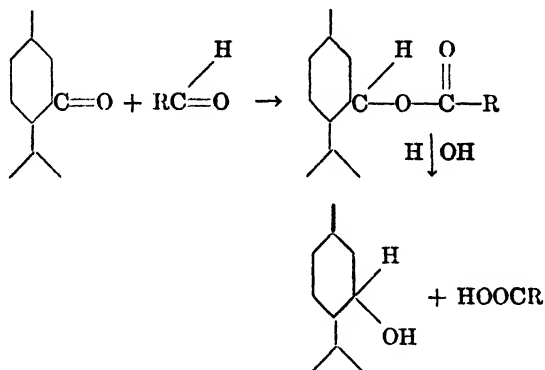
Since acetaldehyde and other aldehydes have been found to accompany compounds of a ketonic nature in the plant kingdom, it may be further suggested that the reaction is of special significance in the plant kingdom.

EXPERIMENTAL.

Two experiments were carried out and in each case the results were concordant.

30 gm. of aluminum ethylate (10) were covered in a 2 liter round bottom flask with a mixture of 154 gm. of freshly prepared *l*-menthone (1 mol) and 106 gm. of freshly distilled benzaldehyde (1 mol) and closed with a stopper bearing a calcium chloride tube. The reaction mixture was allowed to stand at room temperature

with frequent shaking for 4 days. Most of the aluminum ethylate dissolved (11), and the colorless liquid turned light brown.

The flask and its contents were then heated, under a condenser bearing a drying tube, at 175–200° for 30 hours. After cooling, 1½ liters of 5 per cent hydrochloric acid were added.

The oil was taken off and the acid water extracted with ether, and the ethereal solution added to the main portion of the oil. The ethereal solution of the oil was washed with water, 5 per cent sodium carbonate, and again with water until neutral. It was dried over anhydrous sodium sulfate and the ether evaporated.

The residue was then subjected to distillation at 10 mm. pressure, three main portions being collected.

Temperature. °C.	$\mu m.$
73–80	141.5
80–100	11.5
100–250	67.0
Residue.	36.0

The fraction distilling at 100–250°C., which should contain any menthyl benzoate, was again twice distilled at 10 mm., and 32.8 gm. boiling at 165–170° were obtained.

This fraction was a clear, light yellow, odorless and tasteless liquid. Placed in a freezing mixture of ice and salt, it jelled but did not crystallize. Its physical constants were:

Density $\left(\dfrac{25°}{25°}\right)$.. 1.076

$[\alpha]\,_D^{25°}$... −20.24

$[n]\,_D^{25°}$... 1.5585

Saponification equivalent:

1. 2.3742 gm. required 17.84 cc. 0.5 N KOH.
2. 2.3040 "   "   17.64 " 0.5 "   "

Found. (1) 266.1, (2) 261.2. Calculated for $C_{10}H_{19}OCOC_6H_5$, 260.

Saponification.—That the compound in hand was menthyl benzoate was further shown by the saponification products.

The fraction distilling at 165–170°, 10 mm., was saponified by boiling for 6 hours with a solution of 20 gm. of KOH in 250 cc. of alcohol. The greater part of the alcohol was distilled off and the black residue subjected to steam distillation. The volatile part was a clear, colorless liquid and had a decided menthol odor.

The menthol was taken up in ether, dried over anhydrous sodium sulfate, and the ether removed by distillation. 3.8 gm. of menthol were thus obtained. An α-naphthyl urethane and a 3,5-dinitrobenzoate were prepared.

α-Naphthyl Urethane of l-Neomenthol.—3.0 gm. of the alcohol treated with 5.0 gm. of α-naphthyl isocyanate in 20 cc. of heptane were allowed to stand for 24 hours. The insoluble dinaphthyl urea was separated by filtration. Slow evaporation of the solvent yielded the urethane in pure form. M.p. 132°C.[1]

Kjeldahl Analysis.
> 1. 0.2704 gm. substance required 8.29 cc. 0.1 N HCl.
> 2. 0.2355 " " " 7.06 " 0.1 " "

Calculated for $C_{21}H_{27}O_2N$. N 4.30 per cent. Found. (1) 4.29, (2) 4.19.

3,5-Dinitrobenzoate.—The 3,5-dinitrobenzoate was prepared in the usual manner (12). M.p. 94°C.[2]

Since the amount of the ester was very small, a molecular weight determination according to Rast (13) was made.

> 3.131 mg. substance in 29.243 mg. camphor (m.p. 174.5°). M.p. of mixture, 161.5°; hence Δt = 13.0. Calculated for $C_{17}H_{22}O_6N_2$, 340. Found, 329.0.

A simultaneous determination with *l*-menthyl-3,5-dinitrobenzoate prepared according to Cohen and Armes (14) showed the method is applicable to compounds of this nature.

> 5.567 mg. substance in 52.436 mg. camphor. M.p. of mixture 161.5°; hence Δt = 13.0.[3]

Identification of Benzoic Acid.—The alkaline residual liquor from the above mentioned steam distillation was boiled with norit, and the filtered liquid acidified with sulfuric acid. The acid product was filtered and crystallized from water, m.p. 120–121°, and showed no depression when mixed with benzoic acid.

The work is being continued with other aldehydes and ketones, especially those of phytochemical interest.

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[1] The urethane of *l*-menthol melts at 119°, the isomeric form, *l*-neomenthol at 132° (Zeitschel and Schmidt (7)).

[2] The 3, 5-dinitrobenzoate of *l*-menthol melts at 154° (Cohen and Armes (14)).

[3] The micro weighings were made by Mr. L. P. Hallet to whom I here express my thanks.

SUMMARY.

1. *l*-Menthone and benzaldehyde in the presence of aluminum ethylate were condensed to form *l*-neomenthyl benzoate.

2. The applications of the reaction to certain phytochemical reduction processes were discussed.

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THE MANGANESE CONTENT OF PLANT AND ANIMAL MATERIALS.*

By C. W. LINDOW and W. H. PETERSON.

(From the Department of Agricultural Chemistry, University of Wisconsin, Madison.)

Although the fundamental importance of the mineral elements in plant and animal nutrition has long been recognized, there is a marked lack of analytical data on the mineral content of various food materials. In a previous publication (1) attention was called to this deficiency and a report was given of the calcium, phosphorus, and iron content of cabbage grown in various parts of the United States. Within the last 2 months Paul E. Howe (2), Chairman, Subcommittee on Animal Nutrition, National Research Council, has requested research workers to assist in supplying analyses not only for "those inorganic elements ordinarily considered as nutrients—namely, sodium, potassium, calcium, magnesium, sulphur, phosphorus, chlorine, iodine and iron, but also those others the function or effects of which are less perfectly known—namely, manganese, fluorine, bromine, silicon, boron, aluminum, copper, arsenic and zinc."

In the present paper data will be given on the manganese content of 84 samples covering the principal classes of human foods. A number of papers relating to trees and leaves (3-6), to plant food materials (7-23), and to animal food materials (24-28) has already been published on this subject, but in most cases a rather limited number of food materials was analyzed. There is also great lack of uniformity in regard to the methods of analysis employed and the basis on which the manganese is calculated.

The authors fully appreciate that the figures herewith presented do not necessarily represent the manganese content of these

materials generally. Only when an extensive series of samples, grown on different soil types and under different climatic conditions, has been analyzed, will it be possible to state the probable manganese content of any plant foodstuff.

The samples used in this study constituted a representative group of food materials, such as fruits, vegetables, cereals, nuts, and meats bought in the local market. The materials were carefully washed, spread out in thin layers, and left until the water had evaporated. They were then cut in small pieces, dried to constant weight at 100°C., ground in a glass mortar, and bottled. Before taking a sample for analysis the material was dried several hours to remove any absorbed moisture.

The manganese determinations were made according to the periodate method of Willard and Greathouse (29) as described in Methods of Analysis of the Association of Official Agricultural Chemists (30).

Reagents of the highest grade obtainable were used, and all were tested for manganese before being used in the analysis of the samples. Porcelain dishes were used for the ignition of the samples, and these were especially tested. Since the glaze is destroyed when the dish is used several times, more or less of the porcelain may be included with the sample. A test for manganese in the porcelain of the dish was therefore made by placing potassium carbonate in the dish and heating to the fusion point. This treatment removed many times more porcelain than could be taken up by a sample in the regular determination. Three dishes were fused with potassium carbonate and the hydrochloric acid extracts were found to be free from manganese.

The material to be analyzed was thoroughly ashed in a muffle furnace at a temperature of dull redness until the carbon was completely destroyed.

Because of the wide variations in manganese content of the materials used, a single preliminary analysis of each material was made in order to determine the size of sample necessary to produce a depth of color sufficient to be read against the standard. Samples varying in weight from 1 to 15 gm. were subsequently used.

After ashing, the residue was taken up with 25 to 30 cc. of HCl (1:4) and evaporated to dryness in order to render the silica insoluble. The residue was moistened with 2.5 cc. of concentrated HCl and 30 cc. of water. After heating on the sand bath for 1 hour, the sample was filtered, and the insoluble residue washed thoroughly. To the filtrate, which amounted to about 150 cc., 4 cc. of concentrated H_2SO_4 were added, and the whole evaporated on a hot plate to 8 cc. After cooling, $2\frac{1}{2}$ cc. of concentrated HNO_3 were added, and the evaporation continued until white fumes began to come off. After the addition of 50 cc. of water, the solution was heated to dissolve the salts of iron. Then 0.3 gm. of KIO_4 was added, and the solution gently boiled to oxidize the manganese and bring out the full permanganate color. The solution was carefully evaporated to slightly less than 25 cc., transferred to a 25 cc. volumetric flask, and made up to volume. The color was compared with that of a standard permanganate solution in a Duboscq colorimeter.

Duplicate determinations were made in all cases, and a third "recovery" determination was made in some cases.

Preparation of the Standard.—A $KMnO_4$ solution was prepared so as to contain 1 mg. of manganese in 1 cc. of solution. This solution is slightly weaker than a 0.1 N solution. The exact strength of the solution was determined by standardizing against anhydrous $Na_2C_2O_4$. This was the stock solution and from it more dilute solutions were made at the time they were to be used.

Iron salts in solution affect the permanganate color to a slight extent, and because of this fact $Fe(NO_3)_3$ solution containing 1.0 mg. of iron in 1 cc. was added to the standard to give it an iron content about equal to that of the material analyzed. The standard was treated with H_2SO_4 and KIO_4 in exactly the same manner as the sample solution and was also made up to 25 cc. Two or more standards were usually prepared, and the one was chosen which had approximately the same intensity of color as the sample. Usually the standard prism was set at 20 mm. In some cases, where dilute standards had to be used, the prism was set at 30 mm. and in three or four determinations it had to be placed as high as 60 mm. At this extreme limit it is probable that the accuracy of the determination is much reduced.

Recovery of Manganese.—As a check upon the method, a known

quantity of KMnO₄ solution was added to the third sample of many different materials before ashing. This check for recovery of manganese was used in a sufficient number of cases to show that the method was giving satisfactory results.

In Table I will be found detailed data illustrative of the entire procedure. The data for three different materials are given. As will be noted, the size of the samples and their manganese content varied widely, but the recovery of added manganese, ranging from 92.9 to 106.0 per cent, was good in each case.

Manganese Content of Foods.—In Table II are given the moisture and manganese content of 84 food materials. The figures

TABLE I.

Detailed Data Illustrating Procedure for Determination of Manganese in a Few Representative Food Materials.

Sample.	Weight of sample.	Manganese added.	Standard.		Un-known reading.	Manganese.	Recovery of manganese added.	Manganese in sample, dry basis.
			Manganese.	Reading.				
	gm.	mg.	mg.	mm.	mm.	mg.	per cent	per cent
Parsley (average).	2.5		0.20	20.0	20.0	0.20000		0.00800
"	2.5	0.04	0.20	20.0	16.5	0.24240	106.0	
Beans, Lima, (average)........	5.0		0.05	30.0	24.5	0.06123		0.00122
Beans, Lima......	5.0	0.04	0.05	30.0	15.2	0.09868	93.8	
Potatoes (average).......	10.0		0.05	20.0	22.0	0.04545		0.00045
Potatoes..........	10.0	0.04	0.05	20.0	12.1	0.08264	92.9	

for one foodstuff, cabbage, are the averages of twenty-two different samples grown on different types of soil and in different localities.

Manganese varies widely—from none in several species of fish to 0.02162 per cent in northern grown lettuce. Tree fruits grown in temperate regions are low in manganese (0.00040 per cent, average), whereas some tropical tree fruits are high (0.00333 per cent, banana), and others are low (trace, grapefruit). The average percentages of manganese, arranged in ascending order, for some of the principal classes of foods are: 7 animal tissues, 0.00062; 11 bush and vine foods, 0.00178; 10 roots and tubers, 0.00184;

TABLE II.

Manganese Content of Plant and Animal Materials.

Sample.	Moisture.	Manganese in sample, dry basis.	Sample.	Moisture.	Manganese in sample, dry basis.
	per cent	mg. per kg.		per cent	mg. per kg.
Apples, greening.......	82.5	3.9	Grapes, Malaga........	79.6	Trace.
" snow..........	83.9	1.0	Grapefruit............	92.8	"
Apricots..............	40.7	4.8	Halibut...............	76.6	None.
Asparagus............	91.8	12.4	Hog, liver............	68.7	12.2
Banana...............	75.4	33.3	Kohlrabi.............	91.5	13.0
Barley...............	6.8	16.2	Kraut, No. 329........	92.0	7.7
Beans, kidney........	12.5	18.7	" " 332........	91.4	4.0
" Lima..........	12.3	12.2	Kumquat.............	85.0	3.8
" navy..........	14.2	29.6	Lettuce, northern......	95.0	216.2
Beef, kidney..........	79.6	0.5	" southern......	95.0	100.0
" liver............	71.6	8.7	Oats..................	6.2	42.1
" spleen...........	77.1	None.	Onion................	93.7	7.9
" round steak......	75.1	"	Orange peel...........	79.2	5.2
Beet greens (roots)....	87.2	12.8	" pulp and juice.	87.0	Trace.
" " (tops).....	90.3	129.9	Parsley...............	88.2	80.0
Beets.................	82.3	76.2	Peach................	33.4	10.0
"	84.7	35.1	Peanuts..............	1.9	16.0
Blackberries..........	84.1	37.3	Pears, Bartlett........	83.9	4.0
Blueberries...........	81.3	122.4	Peas, green...........	78.6	19.4
Bluefish..............	76.0	None.	Parsnips, scraped......	82.7	2.0
Brussels sprouts.......	87.5	21.3	" unscraped....	75.6	13.9
Cabbage (average of			Peppers, red..........	91.7	18.2
22 samples)..........	92.8	10.6	Pepsin...............	5.4	10.1
Cabbage, red..........	91.4	13.7	Pineapple.............	92.0	133.9
Calf, liver.............		12.0	Pistachio nuts........	4.0	6.6
Carrots...............	90.1	6.0	Pomegranate, seeds....	79.6	8.6
Celery................	94.0	27.1	" skins.....	73.5	16.6
Chard................	91.4	93.5	Potatoes..............	78.2	4.5
Cherries..............	87.5	2.5	" sweet........	72.1	5.4
Codfish..............	81.7	None.	" "	73.3	19.6
Corn, yellow..........	8.6	5.3	Prunes...............	41.2	3.1
Currants..............	28.8	4.4	Quince...............	82.5	Trace.
Dates................	22.3	1.9	Raisins, seeded........	23.6	4.2
Figs..................	37.0	5.5	" seedless.......	27.4	4.7
Gooseberries..........	91.1	4.8	Rye..................	6.4	33.3
Grapes, red California.	83.2	4.9	Salmon...............	75.7	None.
Grape skins, Concord.	77.3	3.9	Shrimp...............	69.6	Trace.
" pulp, " .	72.5	3.0	Spinach..............	91.9	86.5

TABLE II—*Concluded.*

Sample.	Moisture.	Manganese in sample, dry basis.	Sample.	Moisture.	Manganese in sample, dry basis.
	per cent	*mg. per kg.*		*per cent*	*mg. per kg.*
Squash, Hubbard......	90.4	16.7	Walnuts, English.......	3.3	18.6
Strawberries..........	90.9	6.5	Watermelon...........	92.7	2.5
Tangerine.............	86.0	2.6	Wheat................	7.6	37.0
Trout, lake...........	79.0	None.	" bran...........	9.8	101.0
Walnuts, black........	2.0	33.1			

4 nuts, 0.00186; 4 leguminous seeds, 0.00200; 5 cereals, 0.00268; 12 leafy vegetables, 0.00670. Leafy vegetables, which are generally high in all mineral elements, likewise contain the largest amount of manganese. This is about 3 times that contained in the next two groups, cereals and legumes.

Variations are widest among the tree fruits, berries, roots, tubers, and leafy materials. For example, leafy materials range from 0.00106 per cent for cabbage to 0.02162 per cent for northern grown lettuce. Two different samples of the same material have widely different manganese percentages, varying presumably with soil conditions. Northern grown lettuce contains twice as much manganese as southern grown lettuce. The maximum for the twenty-two samples of cabbage analyzed was 0.00159 per cent, and the minimum was 0.00052 per cent. Beets and sweet potatoes show similar variations.

The pineapple is produced in a part of the world where the soils are known to be rich in manganese. On the other hand, many common food materials produced within the state of Wisconsin are still higher in manganese than the pineapple. Lettuce, beet greens (tops), blueberries, and wheat bran are all unusually high in manganese.

SUMMARY.

The manganese content of 84 samples covering the principal classes of food materials is given.

The figures (dry basis) range from none in fish to 0.02162 per cent in northern grown lettuce. The classes of foods in descending order of manganese content are as follows: leafy vegetables,

cereals, legumes, nuts, roots and tubers, bush and vine fruits, animal tissues, and tree fruits.

Different samples of the same foodstuff vary in manganese content. For example a sample of lettuce grown in the north contained more than twice as much manganese as a sample grown in the south. In twenty-two samples of cabbage the manganese ranged from 0.00052 to 0.00159 per cent.

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THE RELATION OF THE HYDROGEN ION CONCENTRATION TO THE TITRATABLE ACIDITY OF MILK.

By PAUL FRANCIS SHARP and T. J. McINERNEY.

(From the Chemical Laboratory of the Department of Dairy Industry, New York State College of Agriculture, Cornell University, Ithaca.)

INTRODUCTION.

A knowledge of the importance of hydrogen ion concentration, as one of the factors in regulating biological and chemical phenomena, leads to the assumption that the hydrogen ion concentration and not the titratable acidity of milk, as such, is the factor which causes some of the properties of milk to change with the acidity. There may be some basis for such reasoning, but the fact that the titratable acidity of milk has been used so successfully in following the changes in milk due to the development of acid, shows that in the majority of cases, at least, these changes are related to the titratable acidity. There must be, therefore, a rather close relationship between the titratable acidity and the hydrogen ion concentration of milk. This paper presents some information as to the general character of this relationship.

A distinctly different relationship exists between the titratable acidity and hydrogen ion concentration of fresh milk, as compared with milk after the production of lactic acid by bacteria has begun.

In a rather general way, at least, Van Slyke and Baker (1) found that high fat content, high solids not fat, and high hydrogen ion concentration of fresh milk were related. In another paper (2) they state: "With the increase of the value of pH, that is, with decrease of hydrogen ion concentration, there is a general tendency for the CO_2 content of the milks to increase and for the degree of acidity, as measured by titration, to decrease. This is not so marked between the pH values of 6.50 and 6.65 as it is above pH 6.65."

Several investigators have considered that the low acidity of certain samples of fresh milk was due to the presence of blood serum or lymph, or to an easier diffusion of the constituents of the blood into the milk.   In considering the evidence which they have obtained on this point, Van Slyke and Baker (1) state:

"This view harmonizes with several facts.   (1) It is in harmony with the changes in composition of the milk; (2) it is in agreement with the hydrogen ion concentration shown by normal milk (pH 6.50 to 6.60), and that shown by blood-serum (about pH 7.60); (3) it harmonizes with the variation found by us in the CO_2 content of milk, normal milk containing about 10 per cent by volume and blood-serum, 65 per cent; and (4) it is in agreement with the increasing number of leucocytes found in the less acid milks."

Baker and Breed (3) found that the increasing pH of fresh milk is associated with an increase in leucocytes and epithelial cells. Also the percentage of samples containing streptococci increased with the pH of the milk.

Data showing some of the variations in titratable acidity of normal fresh milk from cows of the same and different breeds have previously been given by McInerney (4).

Rice and Markley (5) carried out an extensive investigation of the substances contributing to the titratable acidity of fresh milk. They concluded that carbon dioxide accounted for 0.01 to 0.02 per cent of the acidity, calculated as lactic acid, acid citrates 0.01 per cent, casein 0.05 to 0.08 per cent, and albumin less than 0.01 per cent.   They state: "The difference between the total acidity and that due to the sum of the factors just enumerated is attributed to phosphates.   Practically the entire difference in acidity between the various samples was shown to be due to this constituent."   They also show that fresh milk samples with a high titratable acidity show a greater buffer action than samples with low titratable acidity; that is, the change in pH is less on the addition of equivalent amounts of acid and alkali.

Murray and Weston (6) found a general relationship between the pH and the titratable acidity of cream, but the relationship was not very close.   A colorimetric method, which was not very accurate, was used for determining the pH.   They investigated 50 samples ranging in pH from 7.0 to 4.8, and ranging in titratable acidity from 0.08 to 0.61 per cent calculated as lactic acid.   Their data on fresh cream samples and cream samples in which lactic

acid had developed were included in one curve. They point out that a variation in the fat content of the creams used might have been a factor. This would affect both the titratable acidity and the colorimetric determination of the hydrogen ion concentration as they performed it.

<div align="center">EXPERIMENTAL.</div>

Fresh Milk.—In connection with other work considerable data on the hydrogen ion concentration and the titratable acidity of fresh milk were collected. The hydrogen ion concentration was determined electrometrically at 25°C. The titratable acidity was expressed as percentage of lactic acid. On plotting these data it was found that a general relation between the two was indicated, as shown by Fig. 1. Data given by Van Slyke and Baker (2) and by Rice and Markley (5) were also found to agree well with our own data. Several other investigators have given both the titratable acidity and the hydrogen ion concentration of a few samples of fresh milk falling in a very narrow range of acidity. Most of these data are in good agreement with the curve in Fig. 1, and the majority of those which are not were obtained by investigators before the technique for determining the hydrogen ion concentration of milk had reached its present degree of accuracy.

Fig. 1 contains the data for all of the abnormal samples of fresh milk which we could obtain. Many of these samples could hardly be classified as milk but might more properly be called secretions obtained from the udders of cows.

The hydrogen ion concentration expressed as pH ranged from 6.0 to 7.73 and the titratable acidity from 0.05 to 0.50 per cent expressed as lactic acid. The large number of samples falling between pH 6.4 and 6.6 does not indicate that this range is the average for normal milk. This grouping of points is due to our selecting for the determinations, milk which we had reason to believe was high in acidity. The samples with the high pH values possibly became more alkaline after the samples were drawn, due to the loss of carbon dioxide, since no effort was made to prevent its escape. The determinations were usually made within 2 hours after milking, and if it was necessary to hold the samples for a slightly longer time they were cooled at once and
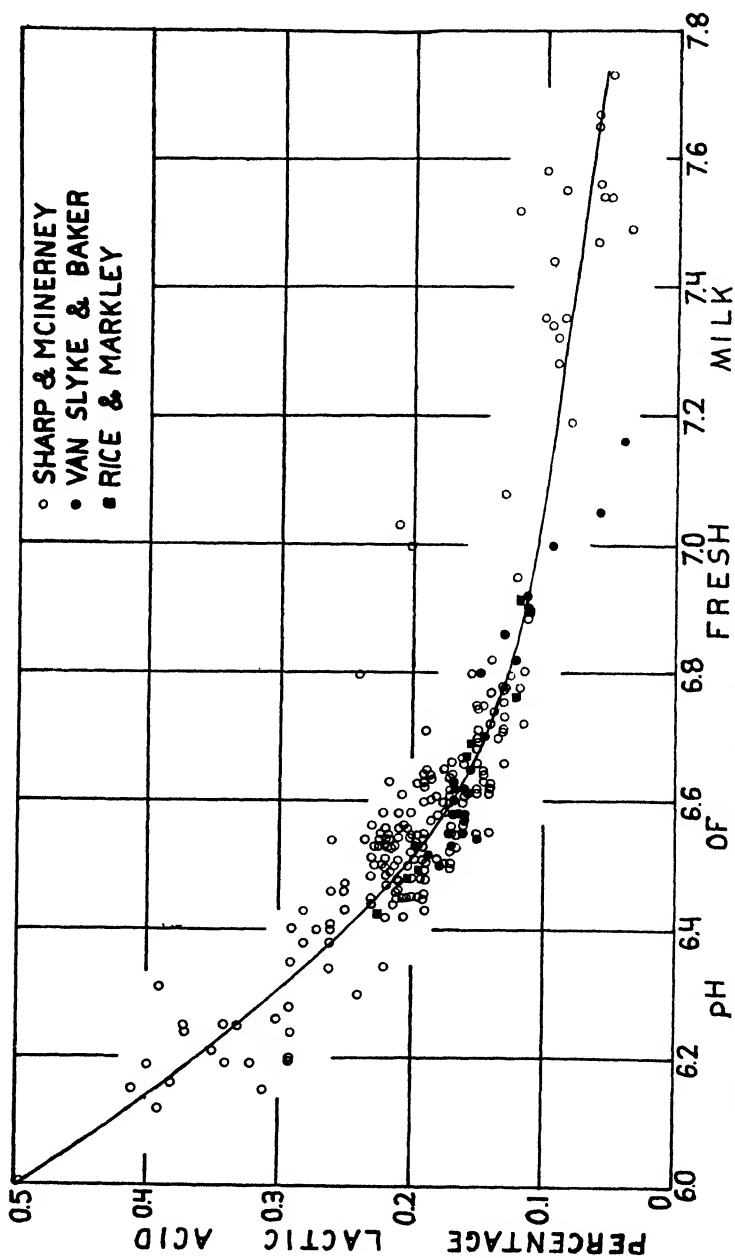
FIG. 1. Relation between the titratable acidity, expressed as percentage of lactic acid, and the pH of fresh milk.

kept in a room at below 5°C.   The samples showing the abnormally high titratable acidity and hydrogen ion concentration were all samples of colostrum milk or milk drawn during the first few days of lactation, but the latter were not always abnormally high in acidity.   The three points which deviate most widely from the curve were obtained with samples from such milk.

Samples of abnormally low acidity in several instances were examined microscopically and were found to contain large numbers of leucocytes.   This observation is in agreement with the results obtained by Van Slyke and Baker (1) and Baker and

TABLE I.

pH of Fresh Milk and Corresponding Titratable Acidity, Expressed as Lactic Acid, Determined from Curve.

pH	Titratable acidity.
6.0	0.50
6.1	0.43
6.2	0.36
6.3	0.30
6.4	0.25
6.5	0.21
6.6	0.165
6.7	0.145
6.8	0.125
6.9	0.115
7.0	0.105
7.1	0.095
7.2	0.090
7.3	0.085

Breed (3).   Several of the abnormal samples were from diseased udders, gargety milk, and from cows which had not been milked for some time.   In spite of the marked abnormality of many of the samples examined, Fig. 1 shows a definite general relationship between the hydrogen ion concentration and the titratable acidity of fresh milk.   Only a very few points are definitely not on the curve.   Below pH 7.0 the pH of fresh milk can be estimated from the titratable acidity with an error which is usually less than 0.1 pH.   The 179 points have 91 per cent of their number falling at least this near the curve.   The average deviation from the

curve is 0.058 pH units.   Table I gives the titratable acidity for
0.1 pH intervals, as estimated from the curve.

The pH of fresh milk below pH 7.0 can be estimated from the
titratable acidity by the use of Fig. 1 with about the same average
error, as it can be determined colorimetrically by the procedure
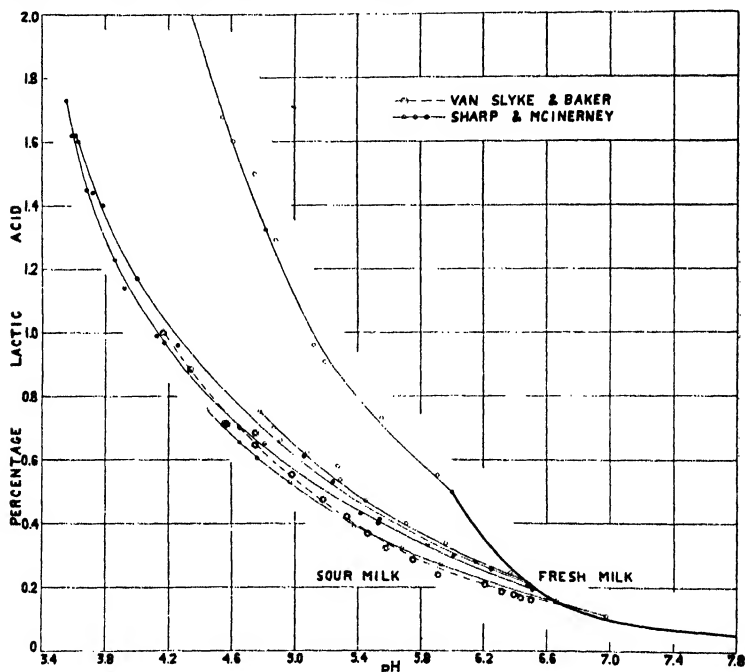described by Sharp and McInerney (7).[1]

FIG. 2. Relation between the titratable acidity, expressed as percentage
of lactic acid, and the pH of sour milk.   The heavy line curve is the curve
for fresh milk.   The sour milk curves start from this base curve.

Sour Milk.—A different relationship between pH and titratable
acidity arises as soon as lactic acid is produced in the milk by
bacteria.   This is indicated in Fig. 2.   The sour milk curves

[1] Sharp and McInerney (7) made the statement that under some condi-
tions the fresh rennet whey did not have the same hydrogen ion concen-
tration as the milk from which it was made.   More careful experiments
indicate that this statement was in error and that whey has practically the
same hydrogen ion concentration as the milk.

start on the fresh milk curve but soon leave it to form a family of curves, which are displaced from each other, depending on the point on the fresh milk curve from which they start.   The slope of the curves for normal milk is fairly uniform, but if the buffer content of the milk is high then the milk changes less in pH for a given unit of lactic acid produced than it does if the buffer content is low.   Fig. 2 also contains data given by Van Slyke and Baker (8) for the development of acid in skim milk inoculated with a pure culture of *Streptococcus lactis* after the milk had previously been pasteurized for 1 hour at 62°C.   It might readily be concluded that if the pH and the titratable acidity of a sample of sour milk are determined, then by the use of Fig. 2 the titratable acidity and the pH of the fresh sample before it soured could be estimated.   In general this is true yet a variation in the kind of bacteria, and more probably in their products, and in the composition of the milk, makes the estimation of the pH and titratable acidity of the fresh milk by the use of Fig. 2 of limited value.

The relationship between the pH and titratable acidity of fresh milk and sour milk makes possible the checking in a rough way at least of the freshness of the milk sample.   It frequently happens (see McInerney (4)) that fresh normal milk from cows of certain breeds is high in titratable acidity, for example, 0.20 per cent. Milk from certain other breeds with an acidity of 0.20 per cent would indicate that considerable lactic acid had developed.   If in addition to the titratable acidity, the pH is also determined, then by means of Figs. 1 and 2 it can be told with approximate accuracy whether or not lactic acid has developed in the milk.   If the sample is fresh the pH of milk with a titratable acidity of 0.20 per cent should be approximately 6.50; if the pH is definitely lower, one can be reasonably sure that the milk is not fresh.   For example, one sample (Fig. 2), when fresh, had a pH of 6.98 and a titratable acidity of 0.11 per cent, but when the titratable acidity had reached 0.20 per cent, due to slight souring, the pH was 6.32; thus the latter point does not fall on the fresh milk curve but in the sour milk region.   Two of the samples, on the other hand, when fresh, had a titratable acidity of 0.19 per cent and a pH of 6.51, and a titratable acidity of 0.20 and a pH of 6.48, respectively. These fall on the fresh milk curve.

Two of the samples of Fig. 2 were inoculated with a high

acid-producing starter while the other samples were inoculated with ordinary starters. Samples which soured naturally give curves the majority of which were very similar to those given in Fig. 2, but there were instances when the curves crossed. The relationship shown in Fig. 2 does not hold for old sour milk.

SUMMARY.

Samples of fresh milk, some abnormal, were obtained which ranged in titratable acidity from 0.50 to 0.05 per cent expressed as lactic acid, and in pH from 6.0 to 7.73.

A relation between the pH and the titratable acidity of fresh milk was found by means of which the pH can be determined from the titratable acidity with an average error of ± 0.06 pH, provided the titratable acidity is greater than 0.10 per cent.

A different relationship exists between the titratable acidity and pH of sour milk, as compared with fresh milk, by means of which fresh milk, which has a high acidity, can generally be recognized.

The titratable acidity of milk is a simple index of the acidity factor, but this investigation indicates that as an adjunct a determination of the pH may in many cases be of great value in determining the condition of acidity of the milk.

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A SIMPLE FORM OF ROTATING DIALYZER.

By NORMAN CHARLES WRIGHT and WILLIAM RULE.*

(From the Department of Dairy Industry, New York State College of Agriculture, and the Department of Physics, Cornell University, Ithaca.)

In order to carry out certain experiments on the distribution of salts on either side of an artificial membrane, it was necessary to devise a simple form of dialyzer, which, while keeping the solutions from contact with air, would at the same time permit adequate stirring.

. A dialyzer of the design described below was finally selected for the work. It presents certain distinct advantages as compared with the forms in general use.

The dialyzer consists of two glass funnels (F), of equal dimensions, the edges of which have been carefully ground on a carborundum wheel, and between which the membrane (M) of collodion, cellophane, or parchment is held. The two funnels are clamped together by means of two wood blocks (B), as shown in Fig. 1. When the funnels are held firmly together by an appropriate adjustment of the four iron bolts running through the wood blocks, the system is air-tight. In selecting funnels for use in the dialyzer, it is advantageous to choose those having stems with fairly wide bore for convenience in filling and emptying the two sides. The ends of the stems are closed by rubber tubing and clamps, or by small stoppers.

The stirring of the solutions in the dialyzer is accomplished as follows: The stems of the funnels are held loosely in two clamps or in bearings of glass tubing (D), and a small, grooved pulley wheel (P) is fixed rigidly to one of them. The pulley wheel is connected by a belt to the shaft of a small motor, by means of

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which the dialyzer may be rotated at any desired speed. In practice a number of dialyzers of this type may be set up and run from the same motor. A battery of four dialyzers has been operated successfully in this way. In filling, a bubble of air should be left on each side of the membrane to facilitate the stirring.

The advantages of this form of dialyzer may be summarized as follows: It is inexpensive and easily assembled from stock labora-
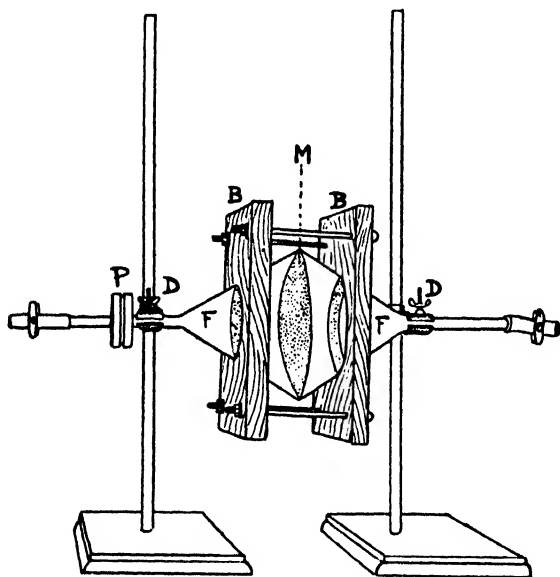

FIG. 1.

tory apparatus. It can be made to hold volumes of solutions varying from 5 or 10 cc. to 1 liter or more. The dialyzer, when rotating, forms in itself a most efficient stirrer. It enables equilibrium to be attained in a comparatively short time, and prevents localized concentration of the diffusing constituents. The solutions are completely protected from atmospheric or bacterial contamination, and, further, the whole dialyzer may, if desired, be immersed in a thermostat. The membranes used are flat, and may consequently be made more easily of definite

thickness and permeability. The dialyzer is easily dissembled for the purposes of cleaning and replacement of membranes.

The apparatus in the form described has been in use for some time by one of the authors and has given satisfactory and consistent results. While it has so far been used only in the simple form described, it seems likely that it would, with suitable modifications, be of considerable value if used on a larger scale for protein purification by dialysis.

STUDIES ON GLUTELINS.

III. THE GLUTELIN OF OATS (AVENA SATIVA).*

BY FRANK A. CSONKA.

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.)

(Received for publication, July 25, 1927.)

Kreusler (1), who was one of the earliest investigators working on the preparation of oat glutelin as at present defined, desired to settle the question whether Johnston's avenin and Norton's (2) preparation obtained by ammonia water extraction were really individual proteins present in the oat kernel. Judging from the way Norton obtained his materials and from their different elementary analyses, Kreusler expressed the view that they probably were mixtures of several proteins. Kreusler called the alkali-soluble protein oat legumin; he obtained this by acid precipitation from the alkali extract of oat flour and by boiling the precipitate with 60 per cent alcohol. The residue which represented oat legumin was practically insoluble in weak acid or alkali. It contained 17.45 per cent nitrogen according to Ritthausen (3), who revised Kreusler's original figure of 17.16.

In 1892, Osborne (4) found that treatment of oat flour with water or salt solution previous to alkali extraction changes the composition of glutelin. He showed that the nitrogen content of the product thus obtained was higher than that of the material prepared by direct alkali extraction. The elementary analyses of his own oat globulin and the alkali-soluble protein were similar. He concluded, therefore, that probably they were essentially the same protein. He thought that Kreusler's oat legumin was also nothing but a modified globulin and that the change was produced by Kreusler, first by extracting the oat flour with water before

* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists held in Rochester, N. Y., April 14 to 16, 1927.

the alkali was added and second by using boiling alcohol.   In recent years Lüers and Siegert (5) and Schryver and Buston (6) published the Van Slyke analysis of oat glutelin; the first named workers prepared the glutelin according to the manner of Osborne, whereas the others used a technique similar in principle to that of Kreusler.   Basing comparison on Van Slyke analysis, Lüers and Siegert (5) found similarity between the glutelin and the crystalline globulin (avenalin).

The author encountered difficulty in the preparation of oat glutelin.   The method that was published for wheat glutelin (7) could not be applied unchanged on account of the insolubility, in a quantitative sense, of oat prolamin in cold alcohol.   If oat flour is extracted with a hot alcohol-water mixture first, 0.2 per cent alkali removes scarcely any appreciable amount of protein from the residue; the heat and alcohol treatment denatures the glutelin, and in such a state it is only slightly soluble in alkali solution.   It is always advisable to avoid the application of heat in the preparation of proteins, because of the formation of secondary products by hydrolysis, or, as in this case, because the proteins themselves may be rendered practically insoluble.

It was found that the addition of very small amounts of ammonium sulfate will separate the glutelin from the prolamin when the two are dissolved in alcohol-alkali.   By repeating this procedure several times a glutelin preparation free from prolamin can be obtained, and then finally the glutelin can be precipitated from its alkaline solution by ammonium sulfate.   From the liquid, after neutralizing with hydrochloric acid, the prolamin could be prepared by dialysis.

Judging from their behavior, the glutelin and the prolamin of oats are likely to occur in some sort of a combination in the seed; whether this is physical (adsorption) or chemical it is hard to say. If oat flour is extracted directly with alkali, the two proteins precipitate together on the addition of ammonium sulfate.   Although the purified glutelin separates at 0.018 ammonium sulfate saturation, there was no precipitation in the original alkali extract until 0.048 to 0.05 ammonium sulfate saturation was reached.   It is interesting to note that the purified prolamin precipitated from its alkaline solution at less than 0.4 ammonium sulfate saturation.

I have compared Lüers and Siegert's (5) data with mine in

Table II. Cystine, arginine, and lysine are higher in my products; on the other hand histidine is higher in their analysis. The low nitrogen percentage, 15.84, of these authors' preparation, points to the presence of impurities (prolamin).

The isoelectric point for the oat glutelin was found to be pH 6.45. Since all the other glutelins published in this series of papers have similar isoelectric points, we may extend our previous list of isoelectric points of proteins (8) for glutelins and say that their isoelectric point is at or near pH 6.45.

Preparation of Oat Glutelin.

The following three different schemes for obtaining a separation of oat glutelin were studied. (1) Extract the oat flour directly with alkali and precipitate glutelin with the addition of ammonium sulfate. As previously stated, this method does not serve the purpose because prolamin and glutelin precipitate together. It was thought that if the prolamin was first eliminated, it would be a simple matter to precipitate the glutelin from its alkali solution by ammonium sulfate. Therefore (2) extract the meal first with 70 per cent alcohol at boiling temperature, because cold alcohol does not eliminate the prolamin quantitatively. Then extract the residue with 0.2 per cent alkali. It was found, however, that very little protein was extractable, because the hot alcohol treatment denatured the proteins, so this arrangement had to be given up. (3) Finally the following plan was tried, and proved successful in yielding a glutelin preparation practically free from impurities. Extract the oat flour with 0.2 per cent alkali, add ammonium sulfate to make it 0.1 saturated. Filter out the precipitate and redissolve it in alkali and alcohol, finally fractionating the glutelin by the addition of ammonium sulfate. Fleurent (9), as mentioned in our first article of this series, separated glutelin by acidification. It has been found, however, that ammonium sulfate produces a better separation without the danger of prolamin admixture.[1]

1 kilo of oat flour prepared in the laboratory was stirred for 4 hours with 6 liters of 0.2 per cent sodium hydroxide solution.

[1] Preliminary experiments showed that there is only one glutelin in the oat kernel; for this reason our first ammonium sulfate precipitation was not higher than 0.1 saturation.

The supernatant liquid was syphoned off and filtered through paper pulp.   The sediment was centrifuged and the liquid was also filtered.   To the combined liquids enough ammonium sulfate

TABLE I.

Elementary Composition of Oat Glutelin in Percentages of Moisture- and Ash-Free Protein.

	per cent
C.	52.60
H.	6.56
S.	0.808
P.	Trace.
N.	17.53
Ash.	0.28

TABLE II.

Distribution of Nitrogen in Oat Glutelin as Determined by the Van Slyke Method Corrected for Solubility of Bases.

	Lüers-Siegert.	Author's.
	per cent	per cent
Amide N.	12.19	13.46
Humin "	2.89	1.42
Cystine N.	1.52	1.99
Arginine N.	14.43	15.30
Histidine N.	7.24	3.49
Lysine N.	4.39	5.45
Amino " of filtrate	52.35	54.71
Non-amino N of filtrate from bases	4.08	2.32

TABLE III.

Amino Acids in Percentages of Moisture- and Ash-Free Oat Glutelin.

	per cent
Arginine.	8.33
Histidine.	2.26
Lysine.	4.98
Cystine.	2.99

was added to make the solution 0.1 saturated.   After standing overnight in the ice box the supernatant liquid was syphoned off, and for more thorough separation the sediment was centrifuged and the liquid discarded.   The precipitate was dissolved in 1 liter

of 0.2 per cent sodium hydroxide and 1700 cc. of 95 per cent alcohol were added, which made the alcohol concentration in the final solution 60 per cent by volume. Part of the glutelin separated at once upon the addition of alcohol, owing to a small amount of ammonium sulfate that remained in the liquid. However, 15 cc. of saturated ammonium sulfate solution were added to be sure that the precipitation was complete. This precipitate represented crude glutelin, and for purification it was redissolved in 0.2 per cent alkali, and filtered. This time when alcohol was added in the same proportion as before very slight precipitation occurred, but after the addition of ammonium sulfate the glutelin separated in flocculent form. The precipitate was washed twice with 60 per cent alcohol and once with distilled water to which enough dilute hydrochloric acid had been added to bring the liquid in which the precipitate was suspended to a pH of 6.4. This precipitate was redissolved in 0.2 per cent alkali and the glutelin was precipitated with ammonium sulfate at 0.018 saturation. The precipitate was washed twice with 0.02 saturated ammonium sulfate, once with acidified water (pH 6.4), then with water, followed by 60 per cent and 95 per cent alcohol. It was dehydrated by absolute alcohol and finally by ether. The yield of glutelin was approximately 1.9 per cent of the oat flour. The results of elementary and Van Slyke analyses are shown in Tables I to III.

SUMMARY.

1. There is probably a chemical or physical combination between glutelin and prolamin in the oat kernel.

2. Only one oat glutelin precipitates from a 0.2 per cent sodium hydroxide solution at 0.018 ammonium sulfate saturation.

3. Van Slyke analysis showed the following percentages: amide N 13.46, cystine N 1.99, arginine N 15.30, histidine N 3.49, lysine N 5.45.

4. The isoelectric point of oat glutelin is approximately pH 6.45.

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SULFUR IN PROTEINS.

III. DERIVATIVES OF *l-* AND *i-*CYSTINE.

A Correction.

By EMIL ABDERHALDEN.

(From the Physiological Institute of the University of Halle, Halle-on-the-Salle, Germany.)

Gortner and Hoffman in dealing on p. 440 of a recent article[1] with di-β-naphthalenesulfon-*l*-cystine refer to an article of mine,[2] in which I report the examination of some organs permeated with cystine crystals. Only small amounts of material were available. In order to identify the amino acid, I coupled the substance with β-naphthalenesulfonchloride by the method of Emil Fischer and Peter Bergell. Since cystine decomposes when heated with an alkali, it was dissolved out of the organ (spleen) in the usual fashion with a minimum of dilute ammonium hydroxide. After the addition of 2 cc. of normal sodium hydroxide, as described on p. 558 of my article, the extract was shaken with 2 gm. of β-naphthalenesulfonchloride in ethereal solution. At the end of each of three successive periods of $1\frac{1}{2}$ hours there were added 2 cc. more of normal sodium hydroxide. I describe the isolation of cystine from the urine with the aid of β-naphthalenesulfonchloride on p. 559. In this instance, also, a large excess of the reagent and, correspondingly, of the alkali was used. Every urine contains some ammonia. The possibility of forming β-naphthalenesulfonamide is therefore always present but if, as in my paper, enough β-naphthalenesulfonchloride and caustic alkali are used, the ammonia is fixed and coupling with the amino acid follows. I cannot understand how Gortner and Hoffman can charge me with having used ammonia instead of sodium hydroxide to accomplish a coupling between cystine and β-naphthalenesulfonchloride. The

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[1] Gortner, R. A., and Hoffman, W. F., *J. Biol. Chem.*, 1927, lxxii, 433.
[2] Abderhalden, E., *Z. physiol. Chem.*, 1903, xxxviii, 557.

Fig. 1.

Fig. 2.

use of a minimal amount of ammonium hydroxide served only to bring about solution of the cystine. Gortner and Hoffman might have saved themselves the trouble of trying to accomplish a reaction between this and β-naphthalenesulfonchloride. The amide must, of course, result.

Di-β-naphthalenesulfon-l-cystine after recrystallization twice from hot alcohol melts at 213–214° (uncorrected). It crystallizes from hot alcohol in the form of delicate needles which coalesce to spherical aggregates (see Fig. 1). β-Naphthalenesulfonamide, similarly purified, melts at exactly 214° (uncorrected). It crystallizes in large spear-shaped needles (see Fig. 2). The amide contains 6.76 per cent N, the di-β-naphthalenesulfoncystine 4.52 per cent. Both compounds are readily soluble in hot alcohol but the amide is dissolved less easily in cold alcohol than the cystine derivative. In hot methyl alcohol the latter compound dissolves with more difficulty than the amide. Both compounds dissolve readily in hot acetic ester. When di-β-naphthalenesulfoncystine is mixed with the amide, the melting point of the former is greatly lowered. I have compared the crystals of my original di-β-naphthalenesulfoncystine with a new preparation of this compound. There can be no doubt, as the analyses then made showed, that I had di-β-naphthalenesulfoncystine in hand. A mixture of the original crystals with a newly prepared di-β-naphthalenesulfoncystine melted at 214° (uncorrected). When I added the amide the mixture melted at 192°.

I regret that Gortner and Hoffman did not read my original article. They would then have seen that ammonia was only used to dissolve the cystine, and that a large excess of β-naphthalenesulfonchloride was used purposely. The appearance of β-naphthaleneamide when ammonia is present has long been known. The presence of a large amount of this substance might interfere with the isolation of di-β-naphthalenesulfoncystine in pure form. It is well, on this account, when quantitative results are sought, first to remove the ammonia. A repetition of the attempt to isolate cystine in the form of its di-β-naphthalene derivative when previously dissolved in just enough aqueous ammonia to bring about its solution was accomplished without difficulty. After recrystallizing once or twice from hot alcohol, the pure cystine derivative was obtained as proved by its crystal form and composition.

SULFUR IN PROTEINS.

III. DERIVATIVES OF *l*- AND *i*-CYSTINE.

A REPLY.

BY ROSS AIKEN GORTNER.

(From the Division of Agricultural Biochemistry, University of Minnesota, St. Paul.)

In reply to the above criticism by Dr. Abderhalden, I wish only to state that in no place in the paper by Gortner and Hoffman do we accuse Dr. Abderhalden of attempting to couple cystine and β-naphthalenesulfonchloride by using only ammonia. We carefully followed Abderhalden's directions as given on p. 558 of his paper, first dissolving 2 gm. of cystine in the minimum amount of ammonia necessary for its solution, then adding 9 cc. of N NaOH and8 5 cc. of ether containing 9 gm. of β-naphthalenesulfonchloride; after shaking 1 hour an additional 9 cc. of N NaOH were added, shaking was continued for another hour, and a final 9 cc. of N NaOH were added. After a further shaking for 1 hour the supernatant ether was removed and the aqueous residue diluted and acidified with hydrochloric acid. The precipitated derivative was filtered off, washed with water, and recrystallized from hot 95 per cent alcohol. On recrystallization it yielded wedge-, or spear-shaped crystals which melted at 213–214°. Analyses for C, H, N, and S, showed this to be β-naphthalenesulfonamide.

Several attempts to prepare the β-naphthalenesulfoncystine from both the *l* and *i* forms of cystine by using ammonia as a solvent for the cystine invariably gave the sulfonamide as the product which was actually isolated.

Abderhalden makes no mention in his original paper of the fact that the amide is formed, or that the ammonia used to dissolve the cystine must be removed before the sulfonchloride is added; neither does he mention the use of heat although in his criticism he states that ammonia was used because "cystine decomposes

when heated with an alkali." At no stage in our experiments was heat employed; in fact, the reaction flask was kept at or below room temperature by occasional cooling.

We prepared the amide by omitting the cystine but keeping all other reagents constant in order to make a mixed melting point determination. The amide mixed with our 213–214° product caused no lowering of the melting point.

Following the directions in our paper we have been unable to prepare a di-β-naphthalenesulfon-l-cystine which melted higher than 203–204° (uncorrected). Repeated recrystallization gave a constant melting product. The crystal form was identical with the photomicrographs as submitted by Abderhalden in the above paper. We cannot explain the failure of our l-cystine to yield a derivative of higher melting point and in light of the above paper by Abderhalden do not doubt that his product is the desired cystine derivative. The fact that his original directions will yield the β-naphthalenesulfonamide as well as the cystine derivative, and the fact that we isolated only the amide which he does not mention, and which has the same melting point as Abderhalden's cystine derivative may perhaps excuse us for questioning Abderhalden's original paper.

PURINE METABOLISM.

I. THE DISTRIBUTION OF URIC ACID IN THE BLOOD AND LYMPH OF THE DOG FOLLOWING THE INTRAVENOUS INJECTION OF URIC ACID.

BY A. A. CHRISTMAN AND H. C. ECKSTEIN.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

Folin, Berglund, and Derick (1) have recently reported a detailed investigation of the distribution of uric acid among the various tissues of the body following the intravenous injection of this substance into various animals (rabbits, dogs, cats, and goats). As a result of these studies it is evident that the injected uric acid left the blood stream very rapidly and that the rate of disappearance was more striking in the case of the dog than in any of the other species. It is likewise clear that uric acid did not accumulate in the tissues in general as evidenced by analyses made of liver, pancreas, spleen, muscles, and intestinal mucosa removed from animals at various periods following the intravenous injection of this substance. Kidney tissue, however, differed from all other tissues studied in that its uric acid content was markedly influenced by the intravenous injection of this compound. Thus while the uric acid content of the kidneys of the control dogs was from 1 to 2 mg. per 100 gm. of kidney tissue, the content of uric acid in the kidneys of the animals which had received uric acid intravenously varied from 38 to 157 mg. for the same weight of tissue. Since only a small portion of the uric acid appeared as such in the urine, either the uric acid was converted in the kidney to some other excretory product or the uric acid was returned to the blood stream. Apparently the urine, which was collected following the administration of uric acid, was not analyzed for allantoin, although this compound is generally considered to be the primary oxidation product of uric acid. As a result of this investigation, Folin

and his coworkers came to the conclusion that the site of uric acid destruction was within the blood.

A review of the literature shows no records of experiments which are concerned with a parallel study of the uric acid content of the blood and lymph following the intravenous injection of uric acid. Inasmuch as it was thought that such an investigation might possibly furnish additional information relative to the rapid disappearance of uric acid following its intravenous administration, it was deemed advisable to make such a study.

Dogs that had been fasted for 24 hours were anesthetized with amytal and the thoracic duct exposed and cannulated. After normal samples of blood and lymph had been secured, uric acid as its lithium salt[1] was injected into the femoral vein. Blood and lymph samples were taken at various intervals. The blood was removed in every case from the femoral artery. As indicated in the tables the samples of blood were taken at the beginning and end of each lymph collection period. The samples of lymph and whole blood were deproteinized according to the method of Folin and Wu. Analyses for uric acid and non-protein nitrogen were made on each sample of whole blood and lymph. The former were made according to the method of Morris and Macleod (3) and the latter by the method of Folin and Wu. In one experiment (Table I) enough blood was removed each time to permit an analysis of both whole blood and plasma.

It is evident from the record of the three experiments recorded in Tables I to III that the normal blood and lymph of the dog contain only traces of uric acid, but that very soon after the injection of uric acid both blood and lymph contain appreciable amounts of this substance. The lymph differs from the blood in that the maximum concentration of the uric acid was reached somewhat later in the former than in the latter. It should be noted, however, that when a high level of uric acid was reached in the lymph, this high level was maintained for a longer period than in the blood. Thus, when the concentration of the uric acid in the blood had fallen to an amount so small that it could not be determined with accuracy, the lymph still contained easily de-

[1] The solution of lithium urate was prepared according to the directions of Koehler ((2) p. 724).

terminable amounts of this substance.   For example, in the experiment recorded in Table I, the uric acid concentration of the lymph collected from 11.37 to 11.57 a.m. was 7.5 mg. per 100 cc.,.

TABLE I.

Experiment 1.

Dog, male; weight 17 kilos.   1200 mg. of amytal given intraperitoneally.

Time.	Volume of sample removed.		Uric acid.		Non-protein nitrogen.	
	Blood.	Lymph.	Blood.	Lymph.	Blood.	Lymph.
	cc.	*cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
11.00 a.m.	29		Trace.		33	
11.00–11.20 a.m.		8.5		Trace.		26
11.26–11.33 "	Injected 85 cc. uric acid solution = 850 mg. uric acid.					
11.37 a.m.	26		3.6 (5.2)*		42	
11.20–11.37 a.m.		12.5		2.7		29
12.02 p.m.	28		1.2 (2.8)		42	
11.37–11.57 a.m.		15		7.5		29
12.25 p.m.	30		1.0 (0.8)		36	
11.57 a.m.–12.22 p.m.		12		3.3		33
p. m.						
1.30	28		Trace (trace).		34	
12.22–1.30		24		1.0		32
2.15	26		Trace (trace).		37	
1.30–2.15		15		Trace.		36
2.18–2.25	Injected 85 cc. uric acid solution = 850 mg. uric acid.					
2.45	30		2.8 (2.6)		42	
2.15–2.45		13		7.7		39
3.22	27		0.9 (0.7)		44	
2.45–3.22		13		3.7		41

* The uric acid values enclosed in parentheses are the concentrations of uric acid in mg. per 100 cc. of plasma.

while the amount in the blood was only 3.6 mg. per 100 cc. at the beginning and had fallen to 1.2 mg. per 100 cc. in a sample taken. shortly after the collection period of the lymph.   Again in a second

experiment (Table II) the content of uric acid in the lymph collected from 12.13 to 12.35 p.m. was 11.4 mg. per 100 cc., whereas the amount found in the blood at the beginning of that period was only 3.8 mg. and had fallen to 1.8 mg. at the end of the period.

TABLE II.

Experiment 2.

Dog, male; weight 25 kilos.   2500 mg. of amytal intraperitoneally.

Time.	Volume of sample removed.		Uric acid.		Non-protein nitrogen.		Total solids. Lymph.
	Blood.	Lymph.	Blood.	Lymph.	Blood.	Lymph.	
	cc.	cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gm. per 100 cc.
11.08–11.36 a.m.		20		Trace.		20	5.5
11.35 a.m.	15		Trace.		28		
11.36 " –12.05 p.m.	Injected 125 cc. uric acid solution = 1250 mg. uric acid.*						
11.36 a.m. –12.05 p.m.		14		0.8		22	5.4
p. m. 12.05	15		10		34		
12.05–12.13		11		12.0		23	6.0
12.13	15		3.8		32		
12.13–12.35		14		11.4		26	5.5
12.35	15		1.8		30		
12.35–12.58		10		5.6		25	5.9
12.58	15		1.2		36		
12.58–1.35		16		2.9		30	5.3
1.35–1.38	Injected 65 cc. uric acid solution = 650 mg. uric acid.						
1.40	15		8.5		41		
1.40–2.05		12		9.0		27	5.4
2.05	15		1.8		36		
2.05–2.40		15		3.3		29	5.5
2.40	15		1.2		38		
2.40–3.20		13		1.8		30	5.7
3.20	15		1.0		37		

* Trouble was encountered in making this injection. The major portion of the uric acid was injected between 12.00 and 12.05 p.m.

In the experiment given in Table III the uric acid was injected at a slow and uniform rate. In this experiment 2380 mg. of uric acid were injected during the course of 56 minutes, whereas in Experiment 1, 850 mg. were injected in a period of 7 minutes and

in Experiment 2, 650 mg. were injected in a period of 3 minutes. Four samples of blood and lymph were taken during the period of the injection. Essentially the same results were obtained in this experiment as in the two preceding ones. During the 16 minutes following the completion of the injection (2.34 to 2.50 p.m., Table III) the uric acid content of the blood had fallen from 6.4

TABLE III.
Experiment 3.

Dog, male; weight 25 kilos. Injected 1600 mg. of amytal intraperitoneally at 11.30 a.m.

Time.	Volume of sample removed.		Uric acid.		Non-protein nitrogen.		Total solids. Lymph.
	Blood.	Lymph.	Blood.	Lymph.	Blood.	Lymph.	
p. m.	*cc.*	*cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*	*gm. per 100 cc.*
1.25–1.38		12		Trace.		18	5.8
1.33	15		Trace.		23		
1.38–2.34	Injected 238 cc. uric acid solution = 2380 mg. uric acid. Injected at uniform rate.						
1.38–1.50		10		2.2		19	5.3
1.55	15		5.0		29		
1.50–2.06		14		7.0		23	Lost.
2.12	15		7.3		33		
2.06–2.18		11		10.0		23	5.3
2.22	15		8.7		34		
2.18–2.30		10		9.2		25	4.7
2.34	15		6.4		36		
2.30–2.50		13		8.8		26	4.8
2.50	15		2.9		33		
2.50–3.06		11		5.5		24	4.9
3.06	15		1.8		30		
3.06–3.23		14		2.6		23	5.2
3.23	15		1.2		30		

mg. per 100 cc. to 2.9 mg. per 100 cc., while the lymph collected in the period beginning at 2.50 p.m. contained 5.5 mg. of this substance per 100 cc.

That the increase in the uric acid content of the lymph cannot be ascribed to an increase in the concentration of this fluid during the course of the experiment, is clear from the figures for the total

solids given in Experiments 2 and 3 (Tables II and III). The values for the non-protein nitrogen of normal lymph agree well with those reported by Arnold and Mendel (4) who demonstrated that the non-protein nitrogen content of blood and lymph did not change appreciably under prolonged amytal anesthesia. The increase in the non-protein nitrogen of the blood and lymph in our experiments is more than can be accounted for by the increase in the uric acid content of these fluids. Since the maximum figures for non-protein nitrogen are not always coincident with the highest values for the uric acid in blood and lymph and in most cases lag slightly behind, one might interpret this to mean that a metabolic product of uric acid was present in the blood.

The theory of Folin, that the blood is the site of uric acid destruction, is based in part at least, upon the failure to find a significant accumulation of this substance in tissue other than the kidney. The evidence presented in this paper demonstrates conclusively that at least part of the uric acid finds its way into the lymph channels. Whether the uric acid which is found in the lymph represents a portion which has been transferred to the lymph from tissues, or whether it represents uric acid which has entered the lymph spaces from the blood and eventually has been transferred to the lymph, cannot be definitely stated. Further studies relative to the fate of uric acid, administered intravenously to dogs and rabbits, are now in progress.

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4. Arnold, R. M., and Mendel, L. B., J. Biol. Chem., 1927, lxxii, 189.

A QUANTITATIVE STUDY OF THE PROBLEM OF THE MULTIPLE NATURE OF VITAMIN B.*

BY H. C. SHERMAN AND J. H. AXTMAYER.

(From the Department of Chemistry, Columbia University, New York.)

Ever since the term vitamin B was applied to the antineuritic vitamin and to the "water-soluble B growth essential," various writers have from time to time warned against acceptance of the implication of identity, and pointed out the possibility of two nutritionally essential substances being covered by this one designation.

Among those who have explicitly advocated the view of the multiple nature of vitamin B and offered experimental evidence in its support, special mention may be made of Emmett and Luros,[1] Funk and Dubin,[2] Levene and Muhlfeld,[3] Kinnersley and Peters,[4] Hauge and Carrick,[5] Laird,[6] Smith and Hendrick,[7] Goldberger and associates,[8] McCollum,[9] and finally Salmon[10] and Chick and Roscoe[11] whose papers have appeared since the experimental work described in the present paper was completed.

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[1] Emmett, A. D., and Luros, G. O., *J. Biol. Chem.*, 1920, xliii, 265.

[2] Funk, C., and Dubin, H. F, *Proc. Soc. Exp. Biol. and Med.*, 1921, xix, 15.

[3] Levene, P. A., and Muhlfeld, M., *J. Biol. Chem.*, 1923, lvii, 341.

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[8] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1926, xli, 297.

[9] McCollum, E. V., Water-soluble vitamins, New York, 1927.

[10] Salmon, W. D., *J. Biol. Chem.*, 1927, lxxiii, 483.

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'Our plan of investigation has been similar in general principle to that of Smith and Hendrick but differs from that recorded in previous publications in that our emphasis has been placed upon the development and use of a more quantitative method to the end that the results thus obtained might be more conclusive as to the multiple nature of vitamin B and might also lay the beginnings of an exact scientific basis for the reinterpretation of vitamin B values of foods into terms of vitamins F and G; the designations which one of us has elsewhere proposed[12] for the two now recognized essentials of the vitamin B complex,—vitamin F being the antineuritic, and vitamin G the other (more thermostable) factor (Goldberger's P-P).

A few words here regarding terminology may perhaps help to avoid further confusion. The letter B in vitamin terminology was originally McCollum's "water-soluble B" and has always connoted the growth-promoting property. Since *both* vitamins F and G are essential to growth, neither alone sufficiently meets our primary conception of vitamin B to make appropriate the assignment of the letter B to either of the now recognized factors into which the vitamin B complex has been resolved. Hence two new designations seemed desirable. Also it seems desirable, until truly chemical names can be assigned, to adhere to the plan adopted by nearly all English-speaking writers and the great majority of others as well, and assign new letters *in alphabetical sequence as the need for each becomes clear.* Hence the term vitamin F (rather than B or B-P) for the antineuritic factor, and vitamin G (rather than B or P-P) for the second factor which several investigators have now shown to be essential, along with the antineuritic factor, for growth, and which Goldberger believes to be also a preventive of pellagra.

<div align="center">EXPERIMENTAL.</div>

Our experiments have all been conducted according to the method developed in this laboratory[13] for quantitative determination of the vitamin B values of foods. This method involves placing healthy young rats when 28 to 29 days old upon a vitamin

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[12] Sherman, H. C., *J. Chem. Ed.*, 1926, iii, 1241.
[13] Sherman, H. C., and Spohn, A. A., *J. Am. Chem. Soc.*, 1923, xlv, 2719. Sherman, H. C., and MacArthur, E. H., *J. Biol. Chem.*, 1927, lxxiv, 107.

B-free diet which is not only adequate but approximately optimal (for growth of rats) in all other respects.   This basal diet consists of: purified casein, 18; Osborne and Mendel[14] salt mixture, 4; butter fat, 8; cod liver oil, 2; corn-starch, 68 per cent.   The corn-starch was such as has been found by Osborne, Wakeman, and Ferry[15] to be free from vitamin B.   The typical results of feeding such rats upon this basal diet alone or with the addition of graduated allowances of vitamin B in the form of certain staple foods have been described in previous papers from this laboratory.[12]

The foods tested in the experiments described in this paper were ground whole wheat, autoclaved yeast, and dried skimmed milk. The wheat was purchased whole, thoroughly freed from dust and other extraneous matter, and ground in the laboratory in a mill used only for this purpose.   Dried bakers' yeast was placed in uncovered Petri dishes to a depth of about 15 mm. and heated in the autoclave at 15 pounds steam pressure for 150 minutes; then, after cooling and drying in the air, it was ground to a powder in a mortar.   The dried skimmed milk was fed as obtained in the market.

These test foods were fed separately and in combination as sources of vitamin B to standard test animals on basal vitamin B-free diet as above described.   The object here was to determine whether merely additive results or evidence of supplementary relationships would be found.   If better growth is induced by the feeding of a mixture of foods as the source of vitamin B than is obtained when double the amount of each is fed separately, then, all other conditions having been properly controlled, the conclusion is justified that the better growth is due to a supplementation of one of the factors of vitamin B by the other, and, therefore, that the multiple nature of vitamin B is established by evidence of a quantitative nature.

Supplementary Relation of Whole Wheat and Autoclaved Yeast.— After much preliminary work to establish a satisfactory level of feeding, a series of experiments was performed in which the sole source of vitamin B was respectively: 0.8 gm. per day of ground whole wheat; or 0.8 gm. of autoclaved yeast; or 0.4 gm. each of

[14] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.
[15] Osborne, T. B., Wakeman, A. J., and Ferry, E. L., *J. Biol. Chem.*, 1919, xxxix, 35.

the wheat and the autoclaved yeast; with negative control experiments in which parallel animals received the vitamin B-free basal diet only.

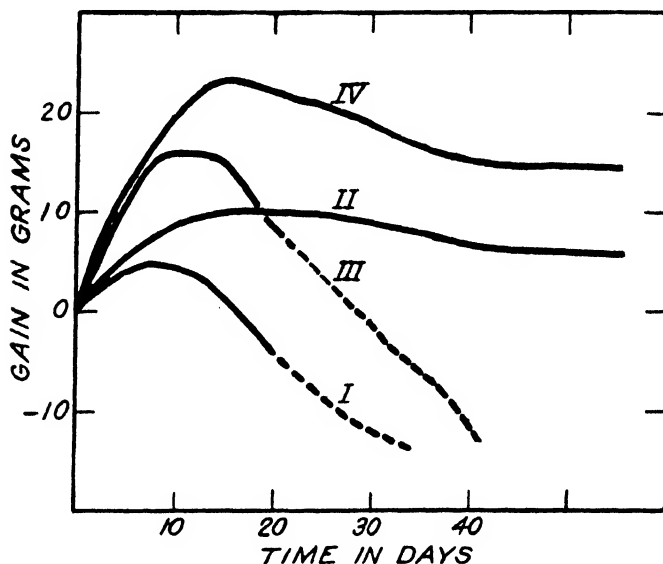The average weight curves are shown in Fig. 1, from which it

Fig. 1. Average weight curves of directly comparable groups of experimental animals (rats of initial age of 28 to 29 days) receiving a basal diet devoid of vitamin B but good in all other respects, and in addition: Curve I, no other food,—these are the negative controls; Curve II, 0.8 gm. daily (except Sundays) of ground whole wheat; Curve III, 0.8 gm. daily (except Sundays) of autoclaved yeast; Curve IV, 0.4 gm. of ground whole wheat plus 0.4 gm. of autoclaved yeast (daily except Sundays). The average weight for each group is represented by a solid line to the point at which the first death occurred among the experimental animals of the group; and from that point onward by a broken line representing the average weight of the surviving experimental animals of the group until all had died (Groups I and III) or until the end of the 8 weeks of the experimental period (Groups II and IV).

will be seen that there was plainly a supplementary relationship and not merely an additive effect when the wheat and yeast were fed together, as compared with the feeding of each separately, as the source of vitamin B.

Simple statistical analysis of the data fully confirms the validity of the conclusion.

Inasmuch as 0.2 gm. per day of dry yeast, carefully prepared, furnishes an abundance of both these factors for the growth of young rats, whereas, the results of feeding 0.8 gm. per day of the autoclaved yeast were such as to indicate the presence of but little vitamin F, it is apparent that the heating in the autoclave had destroyed most, though probably not quite all, of the vitamin F of the yeast, leaving it, however, still relatively rich in vitamin G.

This fact taken with the supplementary relation found to exist between the autoclaved yeast and the wheat shows clearly that whole wheat is relatively rich in vitamin F and is limited in its apparent vitamin B value by its relatively low content of vitamin G.

It therefore seemed feasible (by feeding it in combination with wheat and with autoclaved yeast in turn) to determine whether vitamin F or vitamin G is the growth-limiting factor in milk, which has served as the source of vitamin B in two series of previous experiments in this laboratory.[13]

Supplementary Relation of Wheat and Milk.—By feeding a mixture of 0.4 gm. of ground whole wheat and 0.3 gm. of dried skimmed milk as against double the quantity of each alone, it was found that the milk and the wheat supplemented each other; whereas similar experiments revealed no supplementary relation between the milk and autoclaved yeast. Hence it appears that milk is relatively richer in vitamin G than in vitamin F.

SUMMARY.

Heating bakers' yeast in an autoclave at 15 pounds steam pressure for 150 minutes destroyed very much the greater part, but probably not quite all, of the vitamin F (antineuritic) value of the yeast.

The feeding of such autoclaved yeast to rats receiving *ad libitum* a basal diet good in all other respects but devoid of vitamin B, resulted in a gain in weight for a short period only, after which the average weight curve and food intake of these animals were much like those of parallel animals which received the basal diet only.

That vitamin B consists of more than one factor essential to

the growth of rats is here shown by the supplementation resulting from the feeding of a mixture of ground whole wheat and autoclaved yeast as compared with the feeding of each separately in doubled amount.

Such feeding experiments, carried out quantitatively upon large numbers of young rats have yielded data which seem to establish beyond any reasonable doubt, the significance of the differences in growth which support the view that the vitamin B complex contains more than one substance essential to growth, and also demonstrate the practicability of employing this method to determine which of the two now recognized factors of this complex is the limiting factor in the vitamin B value of a given food; and, conversely, whether the food is relatively richer in the other factor.

Thus the autoclaved yeast which as above shown has been rendered very poor in vitamin F is found to be still a relatively rich source of vitamin G.

Vitamin G is found to be the limiting factor of the vitamin B complex of whole wheat, which is therefore relatively richer in vitamin F than in vitamin G.

Vitamin F is found to be the limiting factor of the vitamin B complex of milk, which is therefore relatively richer in vitamin G than in vitamin F.

These conclusions were further confirmed by systematic examination of the experimental animals for symptoms of polyneuritis.

PROTEINS OF SESAME SEED, SESAMUM INDICUM.

By D. BREESE JONES AND CHARLES E. F. GERSDORFF.

(From the Protein and Nutrition Division, Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.)

The annual world production of sesame seed is approximately 700,000 tons. This seed contains about 50 per cent of oil. The press cake remaining after expression of the oil contains about 40 per cent of protein (N × 6.25), and practically the entire output is used for cattle feed. China and India are the largest producers of sesame seed. Although but little, if any, is grown in the United States, importation of both the seed and the oil has increased during recent years.

The only information heretofore available regarding the proteins of sesame seed is that published by Ritthausen (1) in 1880. He obtained several preparations of protein by extracting the ground press cake with dilute alkali, and with sodium chloride solutions of different concentrations and at different temperatures. The protein obtained from a 10 per cent sodium chloride extract of the sesame meal was redissolved in warm 20 per cent sodium chloride solution. On cooling there separated a small amount of protein in the form of octahedra mixed with a relatively large amount of amorphous material. The only analytical data published with respect to these preparations are figures showing their elementary composition.

By extracting the oil-free meal with 10 per cent sodium chloride solution we have isolated two globulins differing in precipitability with ammonium sulfate, in solubility, elementary composition, and distribution of nitrogen. The α-globulin was obtained as crystals in the form of tetragonal bipyramids (Fig. 1). All preparations of the β-globulin, on the other hand, consisted of amorphous, white powders. It was found that the meal contained approximately 4 times as much α-globulin as β-globulin.

The relatively large amount of the α-globulin obtained as compared with that of the β-globulin is in marked contrast with the proportion of α- and β-globulins obtained from nearly all other sources which we have investigated.   Usually the α-globulin is found in much the smaller quantity of the two.

By heating sodium chloride extracts of the sesame meal to 68°C., a coagulum (Fraction I) containing over 42 per cent of ash was obtained.   The mineral constituents of the ash consisted almost entirely of magnesium, phosphorus, sodium, and potassium. This coagulum contained 12 per cent of nitrogen as calculated on an ash- and moisture-free basis.   It responded positively to the usual tests for proteins.

The distribution of nitrogen in both the globulins and Fraction I was determined by the Van Slyke method, and cystine, tyrosine, and tryptophane were determined colorimetrically.

Material.

The material used in the work here described was obtained from the Oil, Fat and Wax Laboratory of the Bureau of Chemistry and Soils, and consisted of the press cake remaining after expression of the oil from white sesame seed imported from China.   The press cake was ground and freed from the residual oil by extraction with ether.   This oil-free meal contained 6.62 per cent of nitrogen,[1] 12.9 per cent of moisture, and 11.10 per cent of ash.

Preliminary Experiments.

Preliminary 1 hour extractions of 5 gm. portions of the meal with 50 cc. of sodium chloride solutions of various concentrations showed that the maximum amount of nitrogen was removed by 10 per cent sodium chloride solution.   By carrying on the extraction for 2 hours, an amount of nitrogen equivalent to 36.75 per cent of protein (N × 6.25) was removed.   A longer extraction removed less nitrogen, indicating that the protein became somewhat denatured on prolonged contact with the salt solution.

Boiling 70 per cent alcohol removed but a relatively small

[1] The nitrogen determinations in connection with the work described in this investigation were made by Mr. S. Phillips of this laboratory.

amount of nitrogen. Later experiments showed that this nitrogen did not represent prolamin nitrogen.

Strong acidification of the sodium chloride extract of the meal with acetic acid precipitated practically all of the proteins, for no coagulum was formed on boiling the filtrate from the precipitated proteins. This in itself would indicate the absence of an albumin. However, on heating the saline extract to 68°C., a fraction separated containing a high content of ash constituents. Whether the protein residue of this fraction was of the nature of an albumin was not determined.

Dilution of the saline extract of the meal with 10 volumes of water produced an intense opalescence, but caused no precipitation. It is of interest to note, however, that the α-globulin, when separated from the other constituents of the sodium chloride extract of the meal, is readily precipitated from 10 per cent sodium chloride solution by dilution with 10 volumes of water.

Heat coagulation tests made on the slightly acidified (acetic acid) sodium chloride extract of the meal indicated the presence of three distinct protein fractions, coagulating respectively at 68, 88, and 98°C. Heating the unacidified saline extract gave slightly different coagulation temperatures; namely, 74, 84-86, and 97-100°C., respectively. The first fraction (Fraction I) represented the material containing a high percentage of ash constituents, the second fraction represented the β-globulin, and the third fraction, the α-globulin. The coagulation temperatures found in these preliminary tests with the saline extract of the meal do not entirely agree with those noted later for the corresponding isolated and purified proteins.

In order to ascertain as nearly as possible the relative quantities of the different protein fractions present in sesame seed, 100 gm. of the oil-free meal were extracted with successive portions of 10 per cent sodium chloride solution until no more nitrogen was being removed. The three protein fractions were then separated from the joint saline extracts by fractional heat coagulation. The results are given in Table I. Although these figures do not represent the yields of purified proteins, they do, however, indicate more accurately the quantities of the different proteins in sesame seed than would figures based on the yields of the purified

proteins isolated.   The losses involved in the purification of proteins are usually high.

The filtrate from the coagulated proteins was concentrated to a small volume.   Addition of 5 volumes of absolute alcohol to the concentrated liquid produced no precipitation, showing the absence of protein of a proteose nature.

The meal residue remaining after the extractions with sodium chloride solution was further extracted with aqueous 0.25 per cent sodium hydroxide.   By acidifying the alkaline extract with acetic acid, 5.47 gm. of a material were obtained which contained

TABLE I.

Yields of the Proteins Isolated from 100 Gm. of Sesame Seed Meal.

Solvent.	Fraction isolated.	Weight.*	Ash- and moisture-free meal.	Total protein in the ash- and moisture-free meal.
		gm.	*per cent*	*per cent*
10 per cent sodium chloride solution.	Fraction I. (Coagulated at 68°C.)	1.52	2.00	3.68
	β-Globulin. (Coagulated at 84°C.)	5.66	7.45	13.69
	α-Globulin. (Coagulated at 91°C.)	24.35	32.04	58.85
	Total isolated.	31.53	41.49	76.22†

* Corrected for moisture and ash.

† The total protein isolated represents 83.37 per cent of the total crude protein extracted.

4.3 per cent of ash and 10.02 per cent of nitrogen (calculated on an ash- and moisture-free basis).   Whether this material represented an impure glutelin, or some of the globulins that on account of having been denatured had escaped removal by the previous sodium chloride extraction, was not determined.

Preparation of the Proteins.

Fraction I.—This fraction was obtained by heating sodium chloride extracts of the meal to 68°C., whereupon a finely divided white precipitate separated.   The resulting coagulum was washed and dried.   On account of the relatively small yield of this frac-

tion, several preparations thus obtained were united, so as to give a sample large enough for satisfactory purification and analyses. The material was then thoroughly washed over a long period of time with boiling water to constant nitrogen content. The ele-

TABLE II.

Fraction I.

	Elementary composition.*			Composition of ash.			
	I	II	Average.		I	II	Average.
	per cent	per cent	per cent		per cent	per cent	per cent
C..................	44.59	44.56	44.58	Ca.................	0.00	0.00	0.00
H.................	7.31	7.26	7.28	Mg as MgO........	50.29	49.62	49.96
N.................	12.10	12.08	12.09	Na and K as chlorides.............	34.87	34.38	34.48
S..................	0.80	0.80	0.80	P as P_2O_5..........	12.72	12.67	12.70
Ash...............		42.69					
Moisture..........		4.54					

* Calculated on a moisture- and ash-free basis.

TABLE III.

Elementary Composition of the α-Globulin.

Preparation	I	II	III	IV	V	VI	VII	VIII	IX	Average.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
C.................	53.07	53.35	53.32	53.83	53.61	53.08	53.17	52.98	53.47	53.32
H.................	7.02	6.61	6.52	6.59	6.63	6.76	6.93	6.72	6.88	6.74
N.................	18.46	18.28	18.67	18.34	18.66	18.42	18.48	18.34	18.34	18.44
S.................	1.30	1.30	1.26	1.25	1.34	1.33	1.33	1.26	1.18	1.28
Ash..............	0.14	2.36	0.78	1.93	0.26	0.79	0.20	0.21	0.17	
Moisture.........	6.36	5.45	4.76	6.76	8.67	5.78	9.67	8.22	8.72	

* The above figures are averages of duplicate analyses of the different preparations, and represent percentages based on the ash- and moisture-free samples.

mentary composition of this fraction, and the composition of its ash are given in Table II. The properties of Fraction I are given in a following paragraph.

The α-Globulin.—The α-globulin preparations (Table III) were obtained in several ways.

1. The clear, filtered, sodium chloride extract of the meal was made 20 per cent saturated with ammonium sulfate, and the precipitated globulin redissolved in 10 per cent sodium chloride solution. This solution was then dialyzed against running water until free from salts, and the precipitated globulin washed and dried with alcohol and ether in the usual way (Preparation I).

2. The saline extract of the meal, slightly acidified with acetic acid, was heated to 84°C., and the coagulum, consisting of Fraction I and the β-globulin, was removed by filtration. The filtrate was divided into two portions. One portion was heated to 95°C., and the coagulated α-globulin washed and dried (Preparation II).

3. From the other portion of the filtrate above referred to, the α-globulin was precipitated by dialysis (Preparation III).

4. For the remaining preparations an aqueous 10 per cent sodium chloride extract of the meal, slightly acidified with acetic acid, was heated to 73°C., and the coagulated Fraction I filtered off. The filtrate was then made 20 per cent saturated with ammonium sulfate. The α-globulin thus precipitated did not entirely redissolve in sodium chloride solution until the suspension was made neutral to litmus by addition of sodium hydroxide. The solution thus obtained was divided into several portions, from which the α-globulin was separated in different ways. One portion was diluted with 10 volumes of distilled water slightly acidified with acetic acid. The α-globulin separated readily as a flocculent precipitate (Preparation IV).

5. Another portion of the α-globulin solution was diluted with 10 volumes of water, and the resulting precipitate redissolved in 10 per cent aqueous sodium chloride and again reprecipitated by dilution with water. The product was then dissolved in 2 per cent sodium chloride solution at 60°C., and the filtered solution allowed to cool slowly, whereupon a product consisting wholly of microscopic crystals separated. The crystals were washed successively three times with 2 per cent sodium chloride solution, twice with 50 per cent alcohol, twice with absolute alcohol, and twice with ether (Preparation V).

6. A portion of the combined saline mother liquors and washings from the crystals was diluted with 10 volumes of water. The resulting flocculent precipitate was removed and dried in the usual way (Preparation VI).

7. Another portion of the saline mother liquors from the crystalline preparation was allowed to stand for 40 hours at about 10°C. A precipitate having a rather sticky consistency separated. This was dissolved in sodium chloride solution and reprecipitated by water dilution (Preparation VII).

8. Preparation VIII was obtained in the same manner as Preparation VII, with the exception that the water used for diluting the salt solution was slightly acidified with acetic acid.

9. Preparation IX was obtained in the same way as Preparation VIII, except that the saline washings from the crystalline preparation were used instead of the mother liquors.

The elementary composition of these preparations is given in Table III. The uniformity of the analytical results obtained with these preparations made by different methods and precipitated from different fractions, testifies to the homogeneity of the α-globulin.

Direct extraction of the meal with 2 per cent sodium chloride solution at 60°C., without previous removal of Fraction I and the β-globulin, usually yielded a product consisting of small crystals mixed with amorphous material.

Precipitates of the α-globulin which at some time during the course of their preparation had been in contact with dilute acetic acid were not readily soluble in 10 per cent sodium chloride solution until the suspension had been made neutral to litmus by addition of sodium hydroxide.

The β-Globulin.—The β-globulin, which is present in the sesame seed in less than one-fourth of the amount found of the α-globulin, was obtained by fractional precipitation with ammonium sulfate, and by heat coagulation. The preparations referred to in Table IV were obtained as follows. A 10 per cent sodium chloride extract of the meal was heated to 73°C.,[2] and the coagulated Fraction I

[2] Although Fraction I is completely precipitated by heating a 10 per cent sodium chloride extract of the meal to 68°C., it was found that it was necessary to heat the extract to 73°C. in order to be able to isolate the β-globulin by subsequent dialysis of the filtrate from the coagulated Fraction I. This suggests that the higher temperature (73°C.) was necessary to destroy an enzyme present in the sodium chloride extract of the meal which during dialysis affects the β-globulin so as to render it both unprecipitable by dialysis and uncoagulable by heat. For the separation of the α-globulin by dialysis the use of the higher temperature when removing Fraction I was not necessary.

filtered off. Enough ammonium sulfate was added to the filtrate to make the latter 20 per cent saturated with that salt, which caused the precipitation of the α-globulin. The β-globulin was then precipitated from the filtrate by further addition of ammonium sulfate to 60 per cent of saturation. The resulting precipitate was dissolved in water, reprecipitated by dialysis, washed, and dried in the usual way. Preparations I and II were obtained in this way.

Preparations III and IV were obtained by heating the saline extract of the meal to 84°C., after having first removed Fraction I by heat coagulation at 73°C. The coagulated β-globulin was thoroughly washed with hot water and dried in the usual way

TABLE IV.
Elementary Composition of the β-Globulin. *

Preparation.............	I	II	III	IV	Average.
	per cent	per cent	per cent	per cent	per cent
C.................	48.77	48.53	48.62	48.62	48.64
H.................	6.52	6.79	6.39	7.00	6.68
N.................	17.84	17.62	17.38	17.42	17.57
S.................	0.82	0.80	0.79	0.86	0.82
Ash...............	1.05	0.33	9.78	7.11	
Moisture..........	7.37	6.01	5.51	7.53	

* The above figures are averages of duplicate analyses, and represent percentages based on the ash- and moisture-free preparations.

with alcohol and ether. Its elementary composition is given in Table IV.

Properties of the Proteins.

Fraction I.—This fraction is characterized primarily by its high content of ash constituents. Approximately one-half of its ash consists of magnesium as MgO. One-third consists of sodium and potassium as chlorides, and about one-eighth of phosphorus as P_2O_f. It separates from a 10 per cent sodium chloride extract of the meal as a finely divided precipitate on heating to 68°C. The filtrate obtained after removal of the globulins from a salt extract of the meal by acidification with acetic acid, no longer yields a

coagulum on heating.   This shows that the high ash-containing fraction is either precipitated together with the globulins, or is so changed by the action of the acid as to become uncoagulable. It is not precipitated by dilution with water.   It gives the usual protein reactions when subjected to the Millon, biuret, and xanthoproteic tests.   It gave a positive test for carbohydrate with the Molisch reagent.

The α-Globulin.—As in the case of other α-globulins isolated from various seeds, the α-globulin of sesame seed had a rather low range of precipitability with ammonium sulfate.   It is precipitated from
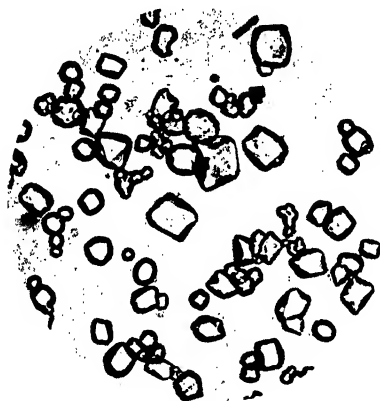
Fig. 1. α-Globulin from sesame seed, *Sesamum indicum.*   × 140. Photomicrograph by G. L. Keenan.

10 per cent sodium chloride solution at 20 to 30 per cent of saturation with ammonium sulfate.

It is readily soluble in 2 per cent aqueous sodium chloride at 60°C., from which it crystallizes on slow cooling in the form of tetragonal bipyramids with very weak double refraction (less than 0.001), the mean refractive indices ranging from 1.546 to 1.548.[3] Photomicrographs of these crystals are shown in Fig. 1.

The α-globulin is readily precipitated from saline solutions by dilution with 10 volumes of water, or by addition of 2 cc. of 1 per

[3] The crystallographic and optical examinations were made by Mr. G. L. Keenan of the Food, Drug and Insecticide Administration.

cent acetic acid per 100 cc. of the saline solution. In a slightly acidified 10 per cent sodium chloride solution, the α-globulin coagulates at 91°C. Like other α-globulins, it is characterized by a relatively high sulfur content as compared with the β-globulin.

The β-Globulin.—The β-globulin was always obtained as a white, amorphous powder. It is not precipitated from sodium chloride solution by dilution with water, even up to 20 volumes; it is, however, completely precipitated from the salt solution by acidification with acetic acid. In very slightly acidified aqueous sodium chloride, it coagulates at 84°C. It is slightly soluble in 2 per cent sodium chloride at 60°C.

The β-globulin is precipitated from 10 per cent sodium chloride solution by addition of ammonium sulfate to 60 per cent saturation.

Analyses of Proteins by the Van Slyke and Colorimetric Methods.

Duplicate samples of 3 gm. each of the proteins were hydrolyzed by boiling for 30 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed with a mixture of ether and amyl alcohol in the usual way. The results of the analyses, corrected for the solubility of the bases, are given in Tables V to IX.

Cystine and tyrosine in the proteins were estimated colorimetrically by the methods of Folin and Looney (2) and tryptophane by the method of May and Rose (3) with slight modifications (4). The results of these analyses, together with the percentages of amino acids and distribution of nitrogen into four groups corresponding to the Hausmann numbers, as calculated from the results of the Van Slyke analyses, are given in Tables VIII and IX.

On account of the unusual character of Fraction I, containing a high content of ash constituents and carbohydrates, it was not expected that perfectly consistent results would be obtained when it was analyzed by the Van Slyke method. As noted in Table IX, the figure obtained for cystine is nearly 3 times that found by the colorimetric method. Inasmuch as it has been

TABLE V.

*Distribution of Nitrogen in Fraction I as Determined by the Van Slyke Method.**

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0086	0.0080	4.49	4.18	4.34
Humin " adsorbed by lime	0.0063	0.0062	3.29	3.24	3.26
" " in ether-amyl alcohol extract	0.0191	0.0190	9.98	9.93	9.95
Cystine N	0.0072	0.0073	3.76	3.81	3.79
Arginine N	0.0439	0.0436	22.94	22.78	22.86
Histidine N	0.0100	0.0102	5.23	5.33	5.28
Lysine N	0.0080	0.0073	4.18	3.81	4.00
Amino " of filtrate	0.0419	0.0415	21.89	21.68	21.78
Non-amino N of filtrate	0.0480	0.0467	25.08	24.40	24.74
Total N regained	0.1930	0.1898	100.84	99.16	100.00

* Each of the two samples analyzed contained 0.1914 gm. of nitrogen, and represented 1.5831 gm. of ash- and moisture-free protein.

TABLE VI.

*Distribution of Nitrogen in the α-Globulin as Determined by the Van Slyke Method.**

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N	0.0486	0.0496	9.39	9.58	9.48
Humin " adsorbed by lime	0.0052	0.0051	1.00	0.99	1.00
" " in ether-amyl alcohol extract	0.0004	0.0006	0.08	0.12	0.10
Cystine N	0.0052	0.0053	1.00	1.02	1.01
Arginine N	0.1359	0.1362	26.25	26.30	26.28
Histidine N	0.0202	0.0205	3.90	3.96	3.93
Lysine N	0.0292	0.0292	5.64	5.64	5.64
Amino " of filtrate	0.2635	0.2642	50.89	51.02	50.96
Non-amino N of filtrate	0.0114	0.0121	2.19	2.34	2.26
Total N regained	0.5196	0.5228	100.34	100.97	100.66

* Each of the two samples of the α-globulin analyzed contained 0.5178 gm. of nitrogen, and represented 2.8050 gm. of ash- and moisture-free protein.

shown that cystine is partially destroyed or altered during prolonged acid hydrolysis, the higher figure obtained by the Van

TABLE VII.

*Distribution of Nitrogen in the β-Globulin as Determined by the Van Slyke Method.**

	I	II	I	II	Average.
	gm.	gm.	per cent	per cent	per cent
Amide N.............................	0.0521	0.0520	10.63	10.61	10.62
Humin " adsorbed by lime.............	0.0060	0.0059	1.23	1.20	1.21
" " in ether-amyl alcohol extract..	0.0018	0.0017	0.37	0.35	0.36
Cystine N.............................	0.0050	0.0053	1.02	1.08	1.05
Arginine N............................	0.1380	0.1373	28.17	28.03	28.10
Histidine N...........................	0.0262	0.0251	5.35	5.12	5.24
Lysine N.............................	0.0206	0.0214	4.21	4.37	4.29
Amino " of filtrate....................	0.2375	0.2390	48.48	48.78	48.63
Non-amino N of filtrate................	0.0072	0.0078	1.47	1.60	1.54
Total N regained....................	0.4944	0.4955	100.93	101.14	101.04

* Each of the two samples analyzed contained 0.4899 gm. of nitrogen, and represented 2.7462 gm. of ash- and moisture-free protein.

TABLE VIII.

Distribution of Nitrogen in Sesame Seed Proteins in Four Groups Expressed in Terms of Percentage of Proteins as Calculated from the Van Slyke Analyses.

Nitrogen.	Fraction I.*	α-Globulin.†	β-Globulin.‡
	per cent	per cent	per cent
Amide.............................	0.53	1.75	1.90
Humin.............................	1.59	0.20	0.28
Basic.............................	4.35	6.81	6.90
Non-basic.........................	5.62	9.82	8.95
Total.........................	12.09	18.58	18.03

* Nitrogen in protein 12.09 per cent.
† Nitrogen in protein 18.46 per cent.
‡ Nitrogen in protein 17.84 per cent.

Slyke method must be ascribed to some complication introduced by the ash constituents of the fraction analyzed. Because of the inconsistently high figure found for cystine nitrogen by the Van

TABLE IX.

*Amino Acids in Sesame Seed Proteins.**

Amino acid.	Fraction I.	α-Globulin.	β-Globulin.
	per cent	*per cent*	*per cent*
Arginine..........................	8.60	15.07	15.58
Histidine..........................	2.36	2.68	3.45
Lysine............................	2.53	5.43	3.99
	4.04†		
Cystine..........................	3.92	1.61	1.61
	1.41‡	1.94‡	1.47‡
Tryptophane‡.....................	2.72	2.77	2.65
Tyrosine‡	4.31	4.72	4.48

* The above percentages were calculated, with the exceptions noted, from the results of the analyses by the Van Slyke method.

† In the calculation of this value for lysine, the cystine percentage obtained by the colorimetric method (1.41 per cent) was used instead of that obtained by the Van Slyke method, and is considered the more accurate.

‡ Determined colorimetrically.

Slyke method, the lysine content was recalculated on the basis of the lower figure for cystine as determined colorimetrically, thus undoubtedly giving a more accurate figure for the lysine.

The unusually high humin nitrogen obtained for Fraction I (Tables V and VIII) is doubtless to be ascribed to the carbohydrate associated with this fraction.

It is of interest to note the close agreement between the cystine figures for the α- and β-globulins as determined by the Van Slyke and by the colorimetric methods (Table IX).

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VITAMIN A POTENCY OF IRRADIATED MILK.

By G. C. SUPPLEE AND ODESSA D. DOW.

(From the Research Laboratories of The Dry Milk Company, New York.)

Numerous investigators have shown that the antirachitic potency of milk can be increased by exposure to the rays of the quartz mercury vapor lamp. However, the desirability of this procedure for enhancing the nutritive properties of milk serving as the sole dietary of the infant, has been questioned, because certain experimental evidence has indicated that the vitamin A of milk is destroyed by irradiation. Titus, Hughes, *et al.* (1) claim that this vitamin is materially decreased in milk irradiated for 20 minutes[1] at a distance of 18 to 20 inches from the lamp. Drummond (2) has likewise pointed out the possibilities of vitamin A destruction in irradiated milk, and he also calls attention to the fact that important, and presumably unfavorable changes are brought about by irradiation as manifested by the fact that milk exposed for as short a time as 5 minutes is highly unpalatable. Spinka (3) states that vitamin A is not destroyed by an exposure of 20 minutes to ultra-violet light, but that the ozone generated by the lamp brings about detrimental results. He states that these results are not evident when air is excluded from the product during irradiation. Steenbock and Coward (4) state that according to their observations, both vitamins A and B have shown marked stability to ultra-violet light. During investigations conducted at this laboratory on milk activated antirachitically with the ultra-violet light, which have extended over a period of 2 to 3 years, observations have not indicated a perceptible destruction of vitamin A in milk irradiated to a degree which imparts marked prophylactic properties for rickets to the product. From these observations as well as those to which reference has been

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[1] Personal communication supplementing the published data.

made, it appears that there is no conclusive evidence indicating that irradiation *per se* destroys vitamin A. It is conceivable that the destructive effect reported by certain investigators has been due to prolonged exposure whereby reactions of a detrimental character have been brought about, such reactions being of a secondary and irrelevant nature in so far as antirachitic activation of the product is concerned.

Data as yet unpublished but now in press, have shown that under suitable conditions, milk, either in liquid or dry form, may be activated to a marked degree by a brief period of exposure. The bone-forming properties of the milk irradiated in this manner are best shown in Table I[2] which summarizes the averages obtained from 217 parallel determinations made with the irradiated and non-irradiated milk.[3]

Since the dry milk in question, irradiated either before or after

TABLE I.

Percentage Ash in Long Bones of White Rats Fed Variable Quantities of Irradiated and Non-Irradiated Reconstituted Dry Milk.

Rachitic ration with:	2 cc. daily.	4 cc. daily.	7 cc. daily.	9 cc. daily.	15 cc. daily.
Non-irradiated dry milk......	43.5	45.5	47.2	52.8	55.6
Irradiated dry milk..........	49.0	53.0	55.1	58.2	60.0

desiccation, was found to possess increased calcifying properties as shown in Table I, the irradiated product has been subjected to further critical studies for the purpose of determining whether or not measurable vitamin A destruction could be detected. The irradiation technique as applied to the product was as follows: For treatment before desiccation the milk was successively exposed in thin films at variable distances from a quartz mercury vapor lamp of the Hanovia type. The minimum exposure distance from the source of light was not less than 12 inches and the maximum distance did not exceed 4 feet. Although the data showing the exact period of exposure at the different distances

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[2] These data are included in an article which will appear in a forthcoming issue of the *Am. J. Dis. Child*, in press.

[3] The product under consideration was a milk containing 12 per cent fat on the dry basis and is known commercially as Dryco brand dry milk.

from the source of light are not available, it is estimated that the total exposure period was for only a period of seconds, and at any rate not over 1 minute.   For treatment after desiccation, the finely pulverized dry milk was likewise exposed successively at variable distances from a quartz mercury vapor lamp of the Cooper Hewitt type.   During the exposure period the powder was subjected to the action of an especially devised agitating apparatus in order to disperse the powder particles thoroughly, thereby permitting an exposure of a very large fraction of the surface area of each particle to the rays of the lamp.   The exposure distances were substantially the same as for liquid milk irradiated, but the average exposure period is estimated to vary from 30 seconds to $3\frac{1}{2}$ minutes.

EXPERIMENTAL.

For the experimental work reported herein, the starvation or vitamin A depletion method was used.   The test animals were vigorous white rats selected from our stock colony, 25 to 28 days old at the time they were used for experimentation.   During the preexperimental period they were allowed to consume the stock colony ration.   While on the test rations they were kept in individual cages with screened bottoms.   The basal ration consisted of casein, 18 parts; Salt Mixture 40 (5), 4 parts; powdered agar-agar, 2 parts; dry yeast, 6 parts; and dextrin, 70 parts.   The casein was freed from vitamin A by extraction 10 days with alcohol and subsequent heating for 1 week at 96°C.

In order to eliminate difficulties in interpreting the results, in so far as the status of vitamin A and the antirachitic factor were concerned, recourse was made to three different procedures.   For one group of test animals no supplementary source of the antirachitic factor was furnished during the vitamin A depletion period; the only antirachitic factor furnished throughout the entire course of the experimental feeding period was that contained in the irradiated or non-irradiated test milks.   In the various accompanying charts this group is always designated by a lot number which begins with the figure 1.   For the second group the antirachitic factor was supplied throughout the test period by irradiation of the basal ration for 20 minutes at 26 inches from the lamp.   The antirachitic factor supplied in this way was therefore

available during the starvation period and supplemented that contained in the experimental milks fed after the vitamin A of the body had been exhausted. In the accompanying charts this group is always designated by a lot number which begins with the figure 2. For the third group the antirachitic factor was supplied throughout the experimental period by direct irradiation of the animals for 3 minutes 6 days a week. In our experience this period of exposure has been found to be just as satisfactory as a longer period. In the accompanying charts this group is always designated by lot numbers which begin with the figure 3.

Vitamin A depletion of the body store was determined by cessation of growth or an occurrence of ophthalmia; both usually occurred simultaneously. The manifestation of ophthalmia can be considered as positive indication of vitamin A depletion, whereas cessation of growth only, may possibly be due to a deficiency of the antirachitic factor, unless this factor is supplied by direct body irradiation or by irradiation of the basal ration. By providing in the experimental plan for different series in which there was an absence of the antirachitic factor in one instance (Series 1), and an adequacy of this factor in others (Series 2 and 3), conclusions regarding the end-point of the vitamin A starvation period are quite definite. For illustration, in those instances where the antirachitic factor supplemented the basal ration and cessation of growth resulted without concurrent ophthalmia, the cessation of growth must have been due to depletion of the body store of vitamin A, whereas, if cessation of growth resulted without the manifestation of ophthalmia in those instances where no supplementary antirachitic factor was furnished in addition to the basal ration it is not definitely known whether the cessation of growth was due to the absence of vitamin A or the absence of the antirachitic factor. With the three series as provided for in the present experimental plan definite assurance regarding the time of vitamin A depletion is possible.

When the growth rate of the individual rats declined, or when ophthalmia occurred, the milk feeding of the test animals was started. The milk as obtained at the laboratory was in dry form, part of which had been irradiated before desiccation and part after desiccation, as described. At the same time the irradiated samples were obtained some of the same milk non-irradiated was

also obtained.   All these milks were obtained during the winter season.   The samples irradiated before desiccation and the non-irradiated check were obtained during the month of January, 1926; the samples irradiated after desiccation and the non-irradiated check samples were obtained early in April of the same year.   The milks were reconstituted to the original fluid milk basis and 5 and 10 cc. quantities of each were fed daily.   Since the milk, when reconstituted to the liquid basis, contained about 1.2 per cent of butter fat, these quantities represented approximately 1.65 cc. and 3.3 cc., respectively, of fluid milk of average fat content.
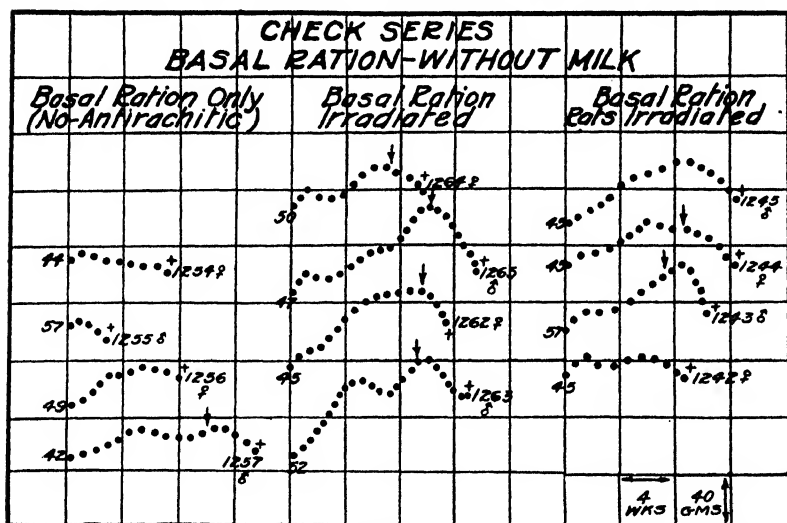
CHART 1.

Computed on the butter fat basis, therefore, the quantities fed represented marginal amounts which might be expected to furnish an adequacy of vitamin A, providing there had been no destruction of this factor during desiccation or irradiation.   It has already been shown that the desiccation of milk by the Just roller process (the method used for drying the milk under consideration) does not cause a measurable destruction of vitamin A (6).

The growth curves recorded in the accompanying charts are presented without selection in order to allow free interpretation of the results.   Chart 1 shows the growth curves of the control
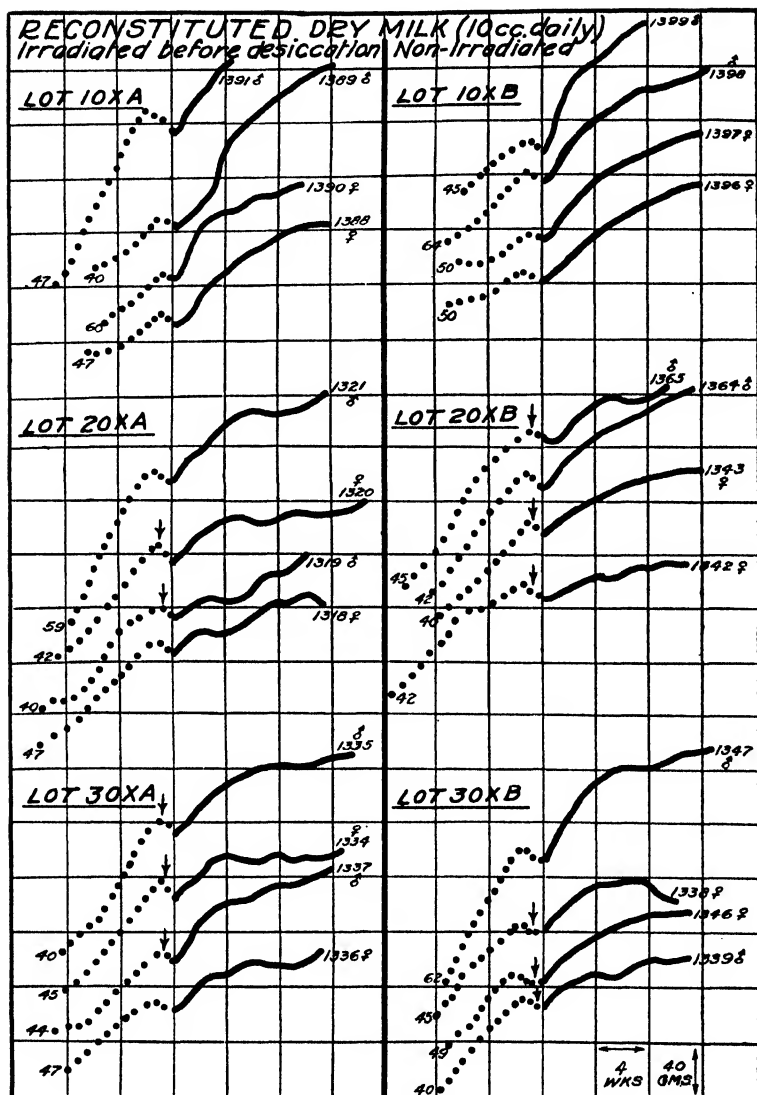
CHART 2.
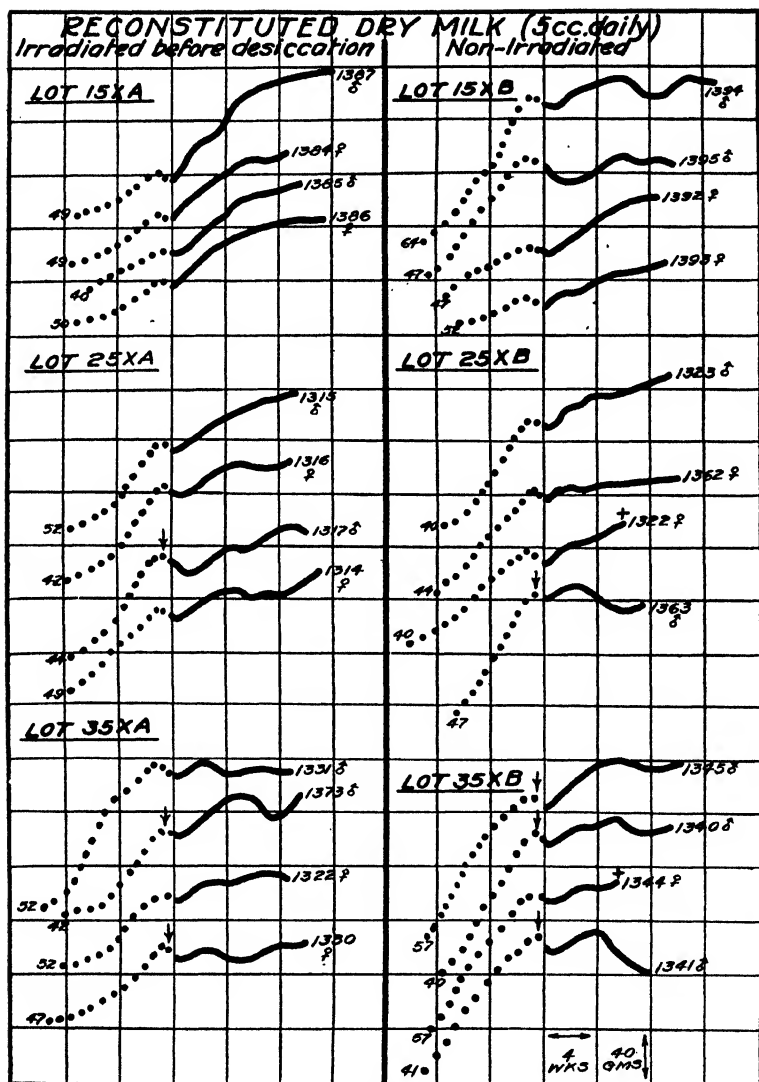
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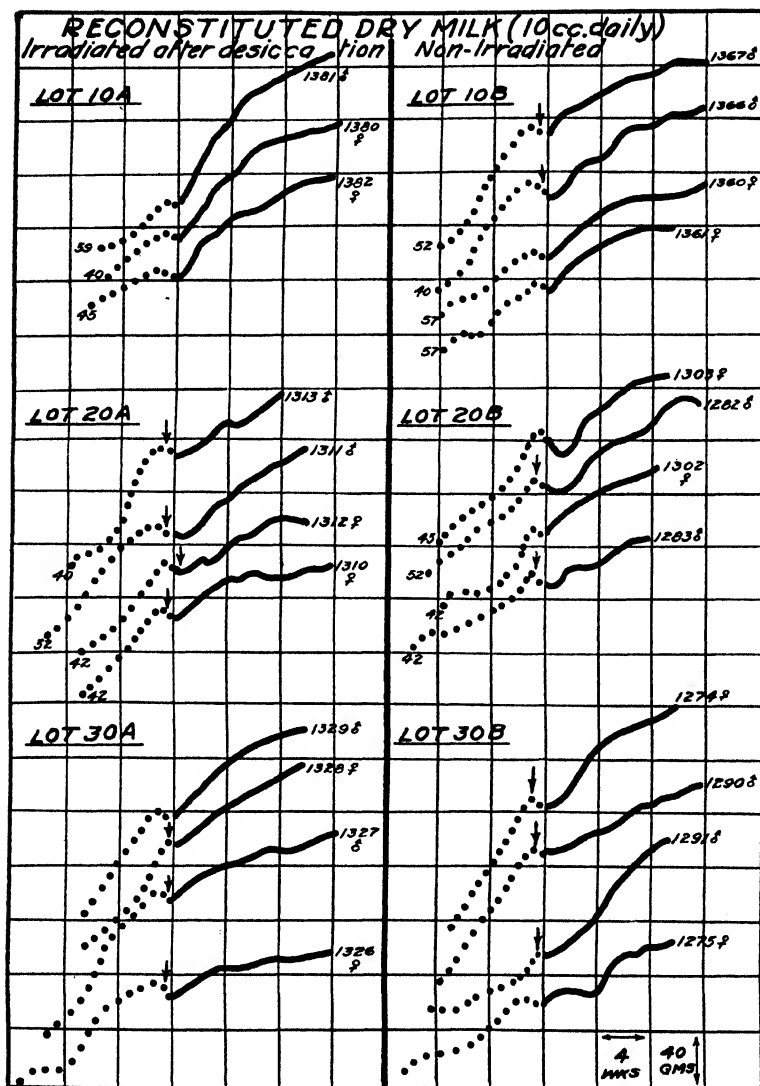
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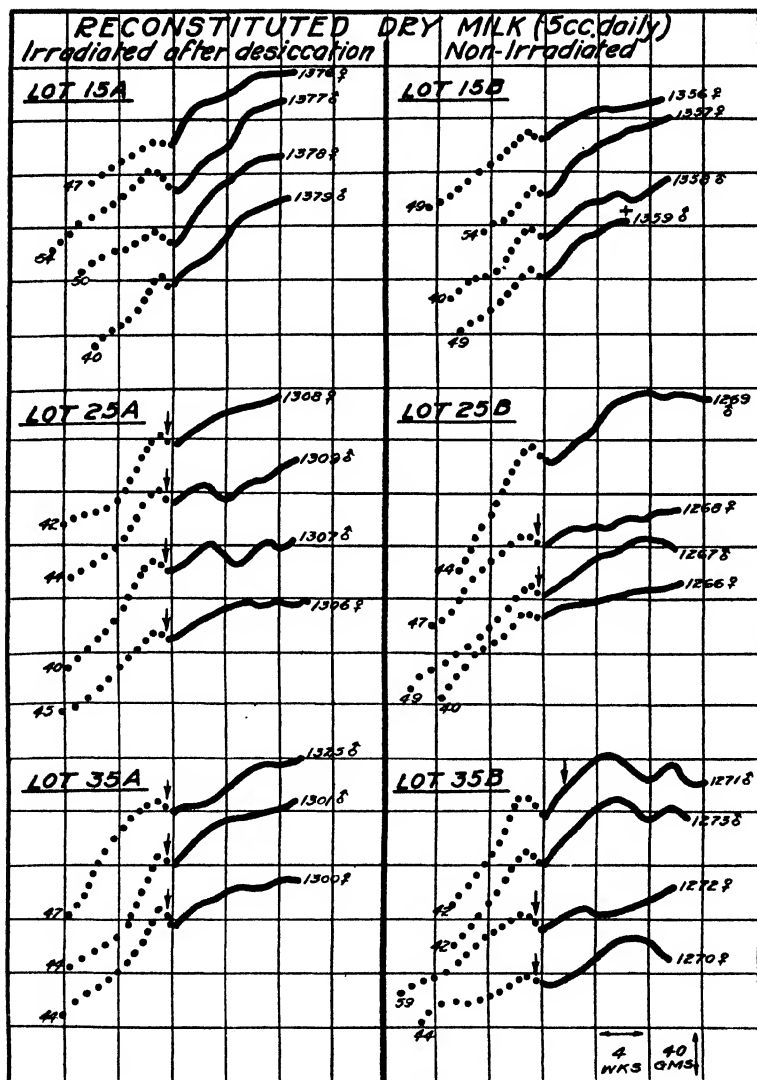
CHART 5.

animals and reveals the influence of the antirachitic factor supplementing the basal ration.   The fact that life is prolonged and the rate of growth is greater when this factor is furnished in the absence of vitamin A in the ration, confirms the observations of other investigators and emphasizes the desirability of furnishing an adequacy of the antirachitic factor in vitamin A studies.   It will be noted that the length of life and the rate of growth of the control animals receiving the antirachitic supplement either by irradiation of the basal ration or by direct body irradiation are

TABLE II.

Average Weekly Gain of White Rats during First 8 Weeks Following Vitamin A Depletion.

Amount fed daily.	Milk irradiated before desiccation.		Milk non-irradiated (check).		Milk irradiated after desiccation.		Milk non-irradiated (check).	
	5 cc. daily.	10 cc. daily.	5 cc. daily.	10 cc. daily.	5 cc. daily.	10 cc. daily.	5 cc. daily.	10 cc. daily.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Series 1, no supplementary antirachitic factor....	6.37	8.87	2.75	8.59	7.72	9.70	4.81	5.81
Series 2, basal ration irradiated....	2.65	3.94	2.33	4.09	2.90	5.25	3.50	5.83
Series 3, rats irradiated...........	1.68	4.62	2.33	4.77	4.25	5.52	4.00	6.87
Average gain for all series............	3.57	5.81	2.47	5.82	4.96	6.82	4.10	6.17
Average gain for Series 2 and 3.....	2.16	4.28	2.33	4.43	3.57	5.38	3.75	6.35

substantially the same, which indicates that either method of supplying this factor appears to be satisfactory for experimental purposes.

The results obtained from the test milks are shown in Charts 2 to 5 inclusive and in Table II.   Table II has been compiled from the weekly weight records of each individual rat in order to facilitate interpretation of the growth curves.   In this table is shown the weekly average of the maximum gains distributed over the first 8 weeks of the milk feeding period.

An inspection of the data shows that the gain resulting from the

feeding of 10 cc. quantities is invariably greater than when 5 cc. were fed.   This is to be expected in view of the marginal quantities of vitamin A carried by these amounts, particularly the 5 cc. quantity.   The significant item for comparison, however, is that there is substantially no difference in the rate of growth of those animals receiving the irradiated and non-irradiated product in those series where an adequacy of the antirachitic factor was furnished from the very beginning of the experimental period.   In the first series where no antirachitic factor was furnished during the vitamin A depletion period, greater growth resulted when the irradiated milk was fed.   While this may be attributed primarily to the antirachitic factor, the relationship also confirms the non-destruction of vitamin A by irradiation, and emphasizes the nutritive enhancement which has accrued to the milk by irradiation.   In the other two series the comparable average gains are substantially the same for the irradiated and non-irradiated milk. The particular exception being in one instance where the difference is 1.3 gm. in favor of the non-irradiated product.   This difference is accounted for by the record of Rat 1291 which gained but very little during the starvation period but did gain at a greater rate than the others of the same group when the milk was fed.

The weekly food consumption records were kept for each rat but since these records show no unexpected relationships they are not included.   The basal ration consumption followed quite closely the trend of the growth curves, decreasing toward the end of the vitamin A starvation period and increasing again when the milk feeding was started.   Coincident with the disinclination to eat during the latter part of the starvation period, kidney and bladder disorders occurred in many of the animals.   This was characterized by the formation of crystallized masses in the urine after excretion; it was usually remedied when milk was fed irrespective of whether the milk was the irradiated or non-irradiated product.   It is possible that this condition is a manifestation of a functional disorder more or less characteristic of vitamin A deficiency.

The ophthalmia which appeared as the result of vitamin A depletion was cured in all instances when milk was fed.   Usually this condition was completely rectified within 10 days to 2 weeks (see Figs. 1 and 2).   Some of the animals receiving the 5 cc.
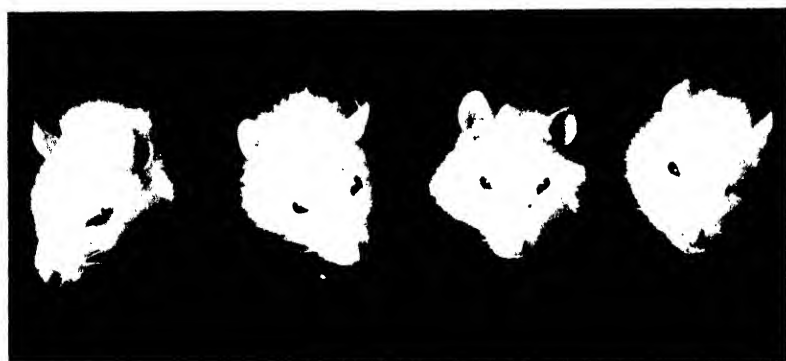
FIG. 1.

FIG. 2.

FIGS. 1 AND 2. In Fig. 1 are shown typical rats at the time of vitamin A depletion, all with severe ophthalmia; in Fig. 2 are shown the same rats 10 days after receiving 10 cc. daily quantities of reconstituted irradiated dry milk. Complete cure has been brought about in each case.

quantities showed a subnormal condition of the eyes after a milk feeding period of 10 to 12 weeks. This condition was noted with equal frequency among animals receiving the irradiated and non-irradiated product. It presumably indicates the marginal protective quantities of vitamin A in the 5 cc. of the low fat milk.

SUMMARY.

Milk irradiated either in dry or liquid form, for short periods under suitable conditions, which had been previously shown to have had imparted to it marked bone-forming properties and prophylactic qualities for rickets, shows, according to the data presented, no evidence of vitamin A destruction, or other toxic effects.

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NOTE ON THE EFFECT OF GLUCOSE ON THE CONDENSATION OF FORMALDEHYDE.

I. THE DETERMINATION OF URINARY SUGAR BY THIS PRINCIPLE.

By FRANCIS B. KINGSBURY.

(From the Biochemical Laboratories of the Metropolitan Life Insurance Company, New York.)

In the development of a new reagent for the colorimetric estimation of sugar in normal urine it was discovered that the reagent could be made many times more sensitive to small amounts of glucose by the addition of formaldehyde. Further experiments showed that this was due to the production of reducing material (formose) by the condensation of the formaldehyde and that the amount of this material so produced was proportional to the amount of glucose present. Thus the catalytic effect of reducing sugar on the condensation of formaldehyde in alkaline solution was made the basis of a new analytical method for the determination of sugar, applicable to urine and blood.

It was the writer's intention not to publish this newly discovered catalytic rôle of glucose (and other reducing sugars) or a description of the new sugar reagent by which this principle was applied until more data had been collected but the recent paper by Schmalfuss (1) containing the discovery that fructose accelerated the condensation of formaldehyde in the presence of magnesium oxide, virtually the same discovery as the writer's, makes it essential to publish this preliminary note.

Experiment Showing That Glucose Accelerates the Condensation of Formaldehyde.

1 cc. of 20 per cent sodium carbonate solution, 0.2 cc. of freshly diluted 10 per cent formaldehyde, and 1 cc. of water are heated for 12 minutes in a test-tube in a boiling water bath. A similar

test, but with the addition to the carbonate and formaldehyde of 1 cc. of 0.001 per cent glucose solution· (0.01 mg.), is made simultaneously. In approximately 7 to 9 minutes the development of a yellow color, the disappearance of the formaldehyde odor, and the advent of the odor of caramel, the characteristic indications that the formaldehyde has been condensed into the group of sugars known as formose, are noted in the tube containing the 0.01 mg. of glucose, but not in the other tube. This yellow solution gives a positive test for sugar with Benedict's qualitative copper sugar reagent. The contents of the other tube are colorless, have a strong odor of formaldehyde, and do not contain sufficient sugar to give a test with Benedict's solution. The presence of 0.01 mg. of glucose has been responsible for the formation of a relatively large amount of formose.

The New Reagent.

37.5 gm. of sodium carbonate, 25.0 gm. of sodium sulfite, and 5.0 gm. of 2,4-dinitro-1-naphthol-7-sulfonic acid[1] are dissolved in approximately 450 cc. of distilled water in an Erlenmeyer flask and boiled very gently for $\frac{1}{2}$ hour. After cooling the deep orange-colored solution is diluted to 500 cc. in a volumetric flask. The solution is now filtered to remove a small amount of insoluble material and bottled. This constitutes the stock reagent from which the final reagent containing formaldehyde is made. 5 cc. of freshly prepared 10 per cent formaldehyde solution in water are added to 100 cc. of the stock reagent solution. Formalin, containing about 40 per cent formaldehyde, is diluted by adding 3 volumes of water in preparing the 10 per cent solution required. The stock reagent solution keeps indefinitely, or at least as long as it has been possible to observe it, a period of several months, but the final reagent containing formaldehyde should be prepared fresh each day, and it is also necessary to use for this purpose formaldehyde that is freshly diluted as recommended above. Most preparations of the reagent stock solution remained clear until consumed but in a few cases spherical burr-like masses of pale

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[1] This substance was obtained from the Eastman Kodak Company Research Laboratory. I wish to thank Dr. H. T. Clarke of that laboratory for information as to the methods used in preparing and purifying this acid.

yellow crystals separated out. These crystals were readily dissolved on gently warming the solution without detectably affecting the quality of the reagent.

Method.

1 cc. of urine measured by an Ostwald pipette is transferred to a small dry Erlenmeyer flask, 9 cc. of distilled water added by Folin blood pipette, and the contents of the flask thoroughly mixed by twirling it. 2 cc. of the 1:10 diluted urine are now transferred by pipette to a test-tube, 150 mm. × 20 mm., graduated at 25 cc.[2] 3 cc. of the sugar reagent are now carefully pipetted into the test-tube and the tube gently shaken, but without inverting, to insure the thorough mixing of the contents, and heated in a rapidly boiling water bath for 15 minutes. Simultaneously 2 cc. each of two glucose standards, 0.005 and 0.01 per cent, are similarly treated. After the 15 minute period of heating, the tubes are cooled by immersion in a large beaker of water for 3 minutes. Distilled water is now added to the 25 cc. mark and the contents of each tube thoroughly mixed. The standard solution most closely matching the contents of the urine tube in color is placed in the left hand cup of the colorimeter and set at 20 mm. 15 cc. of the contents of the urine tube are centrifuged to remove a small amount of white flaky precipitate (probably earthy phosphate) and placed in the right hand cup of the colorimeter. The comparison is made in the usual way. 20 divided by the colorimetric reading times the value in per cent of the standard used, times 10, the number of times the urine was diluted, gives the percentage of sugar present in the original specimen. These directions apply to urines with sugar values ranging from approximately 0.04 to 0.13 per cent.

The dilution of the urine which is the only preparation of the urine necessary in this method of analysis should never be less than 1:4 but should be greater than this when the sugar content is sufficient to permit it since urea and creatinine lower the sugar values if these substances are present in sufficient concentration. By diluting the urine ten times both substances are reduced to

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[2] These tubes may be obtained from the Fales Chemical Company, R. P. Cargille, agent, 74 Cortlandt Street, New York, or from the Emil Greiner Company, 55 Van Dam Street, New York.

non-effective concentrations in most urines. Since glucose standards as low as 0.005 per cent may readily be used and as high as 0.05 per cent, it is not difficult with this extensive range of standards to choose suitable dilutions for diabetic urines as well as for normal urines. The glucose standards are advantageously preserved by means of saturated benzoic acid. Toluene may also be used for this purpose.

The chief advantage of the new method is its simplicity, for on account of the greater specificity of the reagent toward sugars, mere dilution of the urine is the only necessary preliminary step. Thus it is possible to make many more analyses in a day than by any of the previous methods involving the time-consuming but necessary steps in the preliminary treatment of the urine. That mere dilution of the urine in the new procedure is adequate and virtually equivalent to the more elaborate technique of other methods is shown by the fact that sugar values of normal urines by the new method are only slightly higher than by the micro copper method giving the lowest sugar values, the latest method of Folin (2), as modified by Folin and Svedberg (3), and are definitely lower than those given by Benedict's (4) micro copper method.

In one particular the new reagent is strikingly different from copper and picric acid sugar reagents in that it is practically unaffected by homogentisic acid. Thus sugar determinations in alcaptonuric urines can be made by the new method which were impossible by the older methods.

The only substance occurring in urine that is known to have any appreciable effect on the new sugar reagent is urea. Some modification in the new method may, therefore, ultimately be necessary in analyzing specimens particularly rich in this substance, for an addition of 5.6 mg. of urea to 2 cc. of 1:10 diluted urine reduces the sugar value 6 per cent. With most specimens, however, the 1:10 dilution is adequate for obtaining reliable results.

In concluding a description of the newly discovered rôle of glucose and the new reagent by which this principle has been applied to the analysis of urinary sugar it may be mentioned that very sensitive qualitative reagents have been devised along similar lines. One such reagent gave a deep color with less than 0.001 mg. of glucose.

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# THE CATALYTIC OXIDATION OF HYDANTOINS.

By OSKAR BAUDISCH and DAVID DAVIDSON.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

The catalytic oxidation of several organic compounds by sodium pentacyano-ammine-ferroate ($Na_3Fe(CN)_5 \cdot NH_3$) has been previously described (1). The present paper deals with a comparative study of the rate of oxidation of some substituted hydantoins.

Hydantoin itself is represented in Formula I. The derivatives chosen for this research were the following 5-substitution products: methyl (2), dimethyl (3), phenyl (4), and benzyl (5). For comparison, experiments were also made with hydantoic acid ($NH_2CONHCH_2COOH$), glycyl-glycine ($NH_2CH_2CONHCH_2COOH$), and glycyl-glycine anhydride ($NHCH_2CONHCH_2CO$).

The experimental procedure was that described for hydantoin in the previous paper (1).

(3) NH—CO (4)
|
(2) CO
|
(1) NH—CH₂ (5)

I.
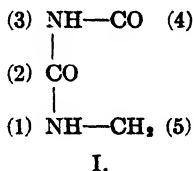
The results are summarized in Fig. 1, from which the following deductions may be drawn.

1. The rate of catalyzed oxidation of 5-substituted hydantoins is a function of the nature of the substituting group. Arranged in descending order of their enhancing action on the oxidation rate, the groups are: phenyl, hydrogen, methyl, benzyl. It is of interest to note that this order is the same as that of the electro-negativity of these groups as determined by Kharasch and Marker (6).

2. Disubstitution of the hydantoin ring in the (5) position (the

case of the dimethyl derivative) completely inhibits oxidation. It is evident, therefore, that the point of attack in the hydantoin nucleus is at the (5) carbon. Since these oxidations are, in general, only apparent in alkaline solution, it is probable that oxidation is preceded by enolization.[1]

$$
\begin{array}{ccc}
\text{NH—CO} & & \text{NH—COH} \\
| \quad\quad | & \rightleftharpoons & | \quad\quad \| \\
\text{CO} \quad | & & \text{CO} \quad \| \\
| \quad\quad | & & | \quad\quad \| \\
\text{NH—CHR} & & \text{NH—CR}
\end{array}
$$
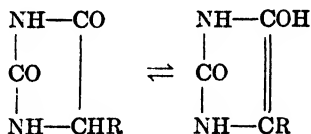
Such an isomerization is, of course, impossible in the disubstituted derivative.
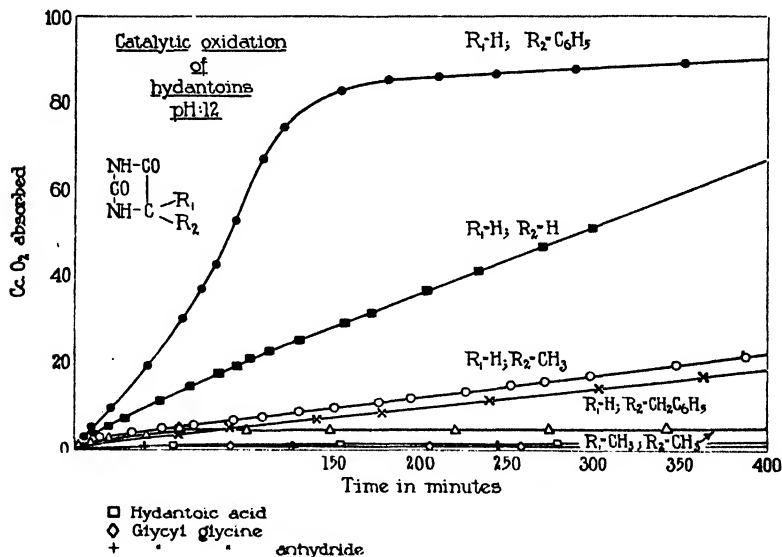
FIG. 1.

3. The hydantoin ring is essential in these oxidations since neither the corresponding open chain derivative, hydantoic acid, nor the corresponding 6-membered ring, glycyl-glycine anhydride, nor its open chain progenitor, glycyl-glycine, was attacked under the same conditions of experimentation.

[1] We are indebted to Dr. P. A. Levene of this Institute for this suggestion.

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A COMPARISON OF EVAPORATED WITH PASTEURIZED MILK AS A SOURCE OF CALCIUM, PHOSPHORUS, AND NITROGEN.*

By ALICE C. WILLARD AND KATHARINE BLUNT.

(From the Nutrition Laboratory of the Department of Home Economics of the University of Chicago, Chicago.)

This study was undertaken for the purpose of securing data in regard to the availability of the calcium, phosphorus, and nitrogen of commercial evaporated milk when used in the diet of human beings, especially children. For comparison, commercial pasteurized milk was fed since it would be the most probable alternative in popular usage.

REVIEW OF LITERATURE.

The authors have found only four sets of experiments bearing directly upon their problems of the comparative physiological values of raw and heat-treated milk, and of these, three were done on animals. Washburn and Jones (1) working with young pigs, found that raw milk gave very slightly better storage of protein than evaporated milk (118 calories and 108 calories) and decidedly better than sweetened condensed milk (59 calories). The breaking strength of the pig's femur was also slightly the greatest on raw; 297 pounds, as opposed to 263 on the evaporated and 200 on the condensed. Magee and Harvey (2), also working with two pigs, obtained poorer retention of calcium, phosphorus, and nitrogen on pasteurized than on fresh or sour milk.

Daniels and Loughlin (3) obtained satisfactory growth in young rats fed quickly boiled milk, or sweetened condensed milk, but not in rats fed slowly pasteurized or evaporated milk. When the pasteurized or the evaporated milk was supplemented by rinsings from the sides of the cans incorporated in a starch paste, or by certain calcium salts, normal growth was secured. In a personal communication in 1924, Dr. Daniels reports noticeably less precipitate in the cans of evaporated milk than at the

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earlier date, and better, though not entirely satisfactory, growth by rats fed the evaporated milk. Improved methods of manufacture in recent years have almost completely checked precipitation in the cans. With babies, Daniels and Stearns (4), observed better gains in weight on quickly boiled than on slowly pasteurized milk, and also better retention of calcium and phosphorus. Their figures for the retention of the elements, however, average rather low on both varieties of milk.

Thus this limited evidence points to the superiority of raw and evaporated milk over pasteurized (pigs being the animal studied), of quickly boiled and condensed over pasteurized (rats), and of quickly boiled over pasteurized (babies).

Clinical experience indicates a general advantage in the use of heat-treated milk for infant feeding, but some disagreement concerning the method of heating (5–8). Pediatricians in the tropics find success in routine use of canned milk (9).

<div align="center">EXPERIMENTAL.</div>

In order to form an estimate of the results of feeding evaporated milk as a source of calcium, phosphorus, and nitrogen for human beings, a metabolism study was made in which a simple diet included evaporated milk diluted 1 : 1 with water during one period, while for another period the same weight of pasteurized milk was used. The calcium, phosphorus, and nitrogen balances were determined in both periods. Every effort was bent toward making the conditions under which the two forms of milk were employed as like each other as possible.

Subjects.—The subjects selected were approximately normal persons, four children and three adults. One of the adults served twice, so eight comparisons of the two forms of milk were secured. Two girls 8 and 12 years of age, and two women served in the first experiment, A. The children came from the stockyards district of Chicago. The younger was stocky, weighed 22.7 kilos, being nearly up to standard in weight. The other was slender, small for her age, and decidedly underweight (29.3 kilos). Neither had been receiving milk regularly nor in appreciable quantities, and in both cases the previous diets had apparently been low in calcium.

In the second experiment, B, two boys served, one just 3 and the other 4 years old, and, as before, two adults. The 3 year old boy

was known to have received a diet very closely resembling the one given in the experiment. His habits of sleep also were excellent. As far as diet is concerned, the 4 year old boy had probably fared satisfactorily, but his allotment of sleep had plainly been inadequate, and his general habits had been irregular, nor was he as well developed as the younger child.

All adults were nutrition students, accustomed to an adequate diet, higher in calcium, phosphorus, and nitrogen than the one used for the experiment but of about the same calorific value.

General Plan.—The two experiments lasted from August 5 to 18, 1925, and from March 20 to April 1, 1926, inclusive, 13 and 12 days respectively. In the first experiment, it was possible to arrange for plenty of sleep, sunshine, and outdoor recreation; but very inclement weather at the close of the second experiment necessitated an undesirable degree of confinement.

Each experiment was divided into two periods of 6 or 7 days each. In Experiment A, the evaporated milk diluted 1:1 with water was fed for Period 1 and the pasteurized milk during Period 2. In Experiment B, this order was reversed. The other foods were kept as uniform in quantity and quality as possible from day to day throughout the experiment.

Milk.—With a view to imitating as closely as possible the household technique in using evaporated milk, the can was cut open and emptied, then filled with water, which was in turn poured into the milk and all well mixed, thus giving the dilution recommended by the manufacturer. No effort was exerted to drain perfectly nor to rinse thoroughly. It might be interesting to note here that in only one can did we find traces of sediment and these were slight. Enough was so prepared each morning to suffice for that day, and combined aliquots of the diluted milk were saved for analysis. Two standard brands of evaporated milk, Pet and Borden's, were purchased on the open market and one tested in each experiment. For comparison, we used the commercially pasteurized milk distributed by one of the large city dairies. The cold diluted evaporated milk and the pasteurized were consumed as a drink or poured over cereal.

Basal Diet.—The basal diet for Experiment A consisted of bacon, banana, ground lean beef, white bread, corn flakes, lettuce, potatoes, fresh peaches, filtered butter fat, and sugar. The food

was planned to furnish the children with an adequate supply of energy, protein, minerals, and vitamins; but for the adults the aim was to furnish sufficient energy and vitamins but a low calcium, phosphorus, and nitrogen intake, fairly close to the calculated minimum requirement for equilibrium. For Experiment B, the basal diet was the same as in Experiment A except that orange juice and canned peaches took the place of bananas and fresh peaches, and cream of wheat was substituted for corn flakes. At no time did any of the subjects appear to tire of the diet nor to find the evaporated milk distasteful. The adults all preferred the pasteurized milk, but the children either voluntarily expressed preference for the evaporated milk or appeared indifferent to any choice.

Tap water was used for drinking purposes. The city water of Chicago has a calcium content of 29 to 36 parts per million. The children drank comparatively little water, being satisfied with the milk for drinking purposes, and it was calculated that, with the exception of one child, the calcium so ingested could not have exceeded 0.1 per cent of the total calcium intake. For M. W., the calculated intake average per day from the water for each period amounted to 15 mg. and has been included in the balances. The adults all used significant amounts of water and calculations based on the average for the city water are included in the intakes.

Collection of Specimens for Analysis.—Collection of materials for analysis did not begin till after a 3 day preliminary feeding of the experimental diet. Then for 3 days, and in Experiment A for 4 days, samples of food and the corresponding urine and feces were saved. After allowing for lag in feces, the second type of milk was substituted and again a 3 day preliminary time allowed to elapse. Then came a final collection period of 3 days. Either ash-free charcoal or analyzed carmine was used for marking stools. Feces were treated immediately with acidified alcohol, dried on a water bath, pulverized, and thoroughly mixed. Collections for the 3 or 4 days were combined for analysis and the determinations made at once or else the material was dried to constant weight at 100°C. and stored. Analyses of the urine were made on aliquots of the 3 or 4 day specimens.

Corn flakes and cream of wheat were analyzed as purchased without further drying. Samples of the ground beef, bread, let-

tuce, riced potatoes, were dried to constant weight at 80°C. in an electric oven, pulverized, and stored in air-tight containers. The raw peaches of Experiment A were canned to preserve them and loss of weight made up by adding distilled water before the fruit was ground to a pulp for analysis. Milk was preserved with formaldehyde, while the commercially canned peaches and the orange juice of Experiment B were analyzed promptly, so storage in a refrigerator provided adequate preservation.

Butter fat and sugar were considered pure foods, while the figures for the calcium of beef and for calcium, phosphorus, and nitrogen of bacon and banana were calculated from Sherman's tables (10), because their contribution to the quantities of these elements in the diet was so slight as to make ordinary variations insignificant. For all other figures on the foods, also for urine and feces, analyses were made.

Methods of Analysis.—Urinary nitrogen was determined by the micro-Kjeldahl method of Koch and McMeekin (11), and this same procedure somewhat modified was applied to determining the nitrogen of foods and feces. For calcium, the standard gravimetric method of McCrudden (12) was used. For foods and feces, ashed samples were prepared and the determinations made upon the whole or an aliquot of the dissolved ash. The ashing was accomplished in an electric muffle furnace at approximately 450°C., according to the Official Methods of the Association of Agricultural Chemists (13). Aliquots of these same ashes were used for determination of phosphorus in food and feces, while for urinary phosphorus Neumann's (14) method of oxidation was employed. The double precipitation method as described by McCandless and Burton (15) was used and the phosphorus finally weighed in a Gooch crucible as magnesium pyrophosphate.

The methods were subjected to a preliminary testing for the recovery of the desired elements from known solutions. The accuracy with calcium was such that from 200 cc. of solution containing 0.0152 gm. of calcium the error was 0.0002 gm., while from 10 cc. of a solution containing 0.0096 gm. of phosphorus the error amounted to 0.0001 gm. These amounts approximate the concentration of calcium and of phosphorus in urine. Further tests were made on solutions containing calcium and magnesium in approximately equal amounts and the same degree of accuracy

as before was secured.   The nitrogen standard, made up from re-crystallized ammonium sulfate, matched exactly that of another worker and the averages of ten matchings with a known solution of urea were in agreement.

All volumetric apparatus was calibrated.   Either duplicate or triplicate determinations were made in every case.   These agreed within 0.8 mg. of calcium oxide or of magnesium pyrophosphate for the gravimetric determinations and within 1 per cent for the nitrogen.

<div align="center">RESULTS AND DISCUSSION.</div>

Table I presents the results of the food analyses, and Tables II and III the daily food intake and the distribution of calcium, phosphorus, and nitrogen among the different foods for one of the older children of Experiment A and for one of the younger children of Experiment B respectively.   The 825 gm. of milk drunk by the older children supplied 90 per cent of the calcium of their diet, 65 per cent of the phosphorus, and 40 per cent of the nitrogen. For the younger children, with 810 gm. of milk, the corresponding percentages are even higher, 95, 78, and 58.

The figures for the daily intake, output, and retention of the three elements by the four children are given in Tables IV, V, and VI, those for the older children coming first.   It should be remembered that the older children were fed evaporated milk first and the younger children the pasteurized.

Calcium Metabolism.—All of the children ingested generous quantities of calcium, in every case up to the gram a day recommended for growing children by Sherman and Hawley (16) and in most cases well in excess of that figure.   Moreover, all retained a considerable amount.   The lowest retention was 0.11 gm. per day and the highest 0.64 gm., the older ones retaining more than the younger.   Sherman and Hawley's children, who were fed 750 gm. of milk daily had, on the average, a calcium intake somewhat lower than our children's and also showed a lower absolute calcium retention.   Their retention per ki o of body weight was close to 0.01 gm., regardless of age, and was thus fairly near the figures for our younger children on the pasteurized milk—0.007 and 0.01—but lower than those for our older children, especially S. K. with her retention of 0.028 and 0.026 gm. per kilo.   When Sherman

TABLE 1.

Food Analyses (Percentage of Edible Portions).

Period.	Beef.	Bread.	Lettuce.	Milk.	Orange juice.	Peach.	Potato.
Calcium.							
A_1		0.037		0.140*		0.013	0.012
A_2				0.135		0.013	0.015
B_1		0.042	0.012	0.116	0.020	0.005	0.010
B_2		0.039	0.013	0.130*	0.014	0.006	0.013
Phosphorus.							
A_1	0.108	0.079		0.094*		0.015	0.046
A_2	0.146			0.098		0.015	0.056
B_1	0.190	0.086	0.021	0.090	0.017	0.010	0.026
B_2	0.184	0.088	0.026	0.098*	0.012	0.014	0.032
Nitrogen.							
A_1	2.94	1.12		0.533*		0.074	0.533
A_2	2.69			0.503		0.077	0.422
B_1	3.14	1.26	0.118	0.560	0.127	0.079	0.309
B_2	3.17	1.28	0.129	0.584*	0.134	0.076	0.354

* Evaporated milk diluted 1:1 with water.

TABLE II.

Distribution of Calcium, Phosphorus, and Nitrogen in Diet of S. K., Experiment A.

Food.	Daily intake.	Calcium.		Phosphorus.		Nitrogen.	
	gm.	gm.	per cent of total	gm.	per cent of total	gm.	per cent of total
Milk	825	1.16	90	0.77	66	4.39	43
Bacon	35	0.00*		0.01*	1	0.59*	6
Banana	70	0.01*	1	0.02*	1	0.15*	2
Beef	75	0.01*	1	0.08	7	2.02	20
Bread	140	0.05	4	0.11	10	1.57	16
Corn flakes	28	0.01	1	0.01	1	0.44	4
Lettuce	30	0.01*	1	0.01*	1	0.06*	1
Peach	180	0.02	1	0.02	1	0.13	2
Potato	280	0.02	1	0.13	11	0.75	7
Butter	25						
Sugar	41						
Total		1.29	100	1.16	99	10.10	101

' From Sherman (10).

TABLE III.

Distribution of Calcium, Phosphorus, and Nitrogen in Diet of J. B.,
Experiment B.

Food.	Daily intake.	Calcium.		Phosphorus.		Nitrogen.	
	gm.	gm.	per cent of total	gm.	per cent of total	gm.	per cent of total
Milk......................	810	1.05	94	0.80	80	4.73	59
Bacon.....................	10	0.00*		0.01*	1	0.17*	2
Beef......................	35	0.00*		0.06	6	1.11	14
Bread.....................	60	0.02	2	0.05	5	0.77	10
Lettuce...................	10	0.00		0.00		0.01	
Orange juice...............	100	0.01	1	0.01	1	0.13	2
Peach.....................	160	0.01	1	0.02	2	0.12	1
Potato....................	120	0.02	2	0.04	4	0.42	5
Wheat.....................	28	0.01	1	0.02	2	0.50	6
Butter	20						
Sugar.....................	8						
Total........................		1.12	101	1.01	101	7.96	99

* From Sherman (10).

TABLE IV.

Calcium Balance (Daily Average).

Subject.	Kind of milk.	Total in-take.	Urine.	Feces.	Total out-put.	Retention.		
		gm.	gm.	gm.	gm.	gm.	per cent of intake	gm. per kg.
M. W. (Experiment A.)	Evaporated.	1.33	0.05	0.77	0.82	0.51	38	0.017
	Pasteurized.	1.28	0.09	0.82	0.91	0.37	29	0.013
S. K. (Experiment A.)	Evaporated.	1.29	0.15	0.56	0.71	0.58	45	0.026
	Pasteurized.	1.24	0.14	0.46	0.60	0.64	52	0.028
J. B. (Experiment B.)	Evaporated.	1.12	0.02	0.89	0.91	0.21	19	0.014
	Pasteurized.	1.01	0.02	0.88	0.90	0.11	11	0.007
B. P. (Experiment B.)	Evaporated.	1.12	0.01	0.91	0.92	0.20	18	0.012
	Pasteurized.	1.06	0.02	0.88	0.90	0.16	15	0.010

and Hawley increased the milk intake for three of their children,
12 years, 5 years and 2 months, and 4 years and 2 months, they
had four pairs of experiments for each child where the intakes of

calcium, 0.929 to 1.273 gm., were not far from those of our children. On these intakes, their 12 year child retained more total calcium and

TABLE V.

Phosphorus Balance (Daily Average).

Subject.	Kind of milk.	Total intake.	Urine.	Feces.	Total output.	Retention.		
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*per cent of intake*	*gm. per kg.*
M. W.	Evaporated.	1.26	0.57	0.45	1.02	0.24	19	0.008
(Experiment A.)	Pasteurized.	1.26	0.59	0.51	1.10	0.16	13	0.005
S. K.	Evaporated.	1.18	0.68	0.17	0.85	0.33	29	0.015
(Experiment A.)	Pasteurized.	1.18	0.73	0.30	1.03	0.15	13	0.007
J. B.	Evaporated.	1.02	0.45	0.37	0.82	0.20	20	0.013
(Experiment B.)	Pasteurized.	0.94	0.48	0.35	0.83	0.11	12	0.007
B. P.	Evaporated.	1.02	0.39	0.36	0.75	0.27	26	0.016
(Experiment B.)	Pasteurized.	0.98	0.41	0.42	0.83	0.15	15	0.009

TABLE VI.

Nitrogen Balance (Daily Average).

Subject.	Kind of milk.	Total intake.	Urine.	Feces.	Total output.	Retention.		
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*per cent of intake*	*gm. per kg.*
M. W.	Evaporated.	10.85	7.48	1.16	8.64	2.21	20	0.08
(Experiment A.)	Pasteurized.	10.61	7.60	1.05	8.65	1.96	18	0.07
S. K.	Evaporated.	10.09	7.99	0.92	8.91	1.18	12	0.05
(Experiment A.)	Pasteurized.	9.88	7.50	1.15	8.65	1.23	12	0.05
J. B.	Evaporated.	7.96	5.21	0.85	6.06	1.90	24	0.13
(Experiment B.)	Pasteurized.	7.70	5.62	0.68	6.30	1.40	18	0.09
B. P.	Evaporated.	7.98	5.08	0.83	5.91	2.07	26	0.22
(Experiment B.)	Pasteurized.	7.95	5.83	0.62	6.45	1.50	19	0.09

more per kilo than our child of the same age, but not so much per kilo as our 8 year child. Their 4 year old retained more than

our child of that age.   The results from other investigators which Sherman and Hawley summarize with their own in their Table V show an average calcium retention of 0.011 gm. per kilo, which is about the same as our younger children, and for 9 to 14 years, 0.009 gm., a figure decidedly lower than those of our older children.

On the basis of such a comparison, then, we seem justified in concluding that the calcium intake of our children was well up toward the optimum and that the retentions secured were also good with both kinds of milk used.

Three of our four children showed higher retention from the evaporated milk, this difference being marked in two cases.   One child, S. K., who retained more calcium in both periods than any of the other children, maintained a somewhat greater retention from the pasteurized milk.   Under these circumstances, we seem further justified in the conclusion that evaporated milk is at least as satisfactory as a source of calcium for children as is pasteurized and possibly somewhat better.

In point of view of age, our older children compare nicely with the 2 little girls 10 and 12 years old studied by Chaney and Blunt (17) who, however, used a much lower calcium intake and, as would be expected, secured a much lower retention.   Their highest intake amounted to but 0.75 gm. per day, which gave a retention of 0.24 gm., while our lowest retention for these older children was 0.37 gm. on an intake of 1.28 gm. per day.

In feeding infants, Daniels and Stearns (4) apparently secured very extreme results, for their figures, calculated to calcium content, show balances ranging from −0.011 to 0.039 gm. per kilo per day.   It also seems surprising that five negative balances should appear out of a total of 18.   All of the children studied by Sherman and Hawley, the two reported by Chaney and Blunt, and the four investigated by us, with ages ranging from 3 to 13 years, showed positive balances.

Phosphorus Metabolism.—The phosphorus intake also amounted to approximately a gm. per day, and without exception, both by actual weight and by percentage of intake, the retention was decidedly higher with the evaporated milk, these differences amounting to between 0.08 and 0.18 gm. per day (Table V). These results, also, would suggest an advantage in the use of evaporated milk as a source of phosphorus.

Nitrogen Metabolism.—The daily intake of protein averaged somewhat over 2 gm. per kilo per day for each child, if 6.25 be used as factor, so was fairly close to the standard recommended by Sherman (10) and by Holt (7). The statements made concerning the calcium retention of the children repeat themselves in the results of the nitrogen determinations. One child, S. K., had a slightly higher retention with the pasteurized than with the evaporated milk. The figures with the other three children are considerably better with evaporated milk (Table VI).

That the differences are in favor of evaporated milk in ten out of twelve balances reported for the children of this study is thus apparent. A partial explanation of these results might be that the intakes also were usually higher. These differences in intake are, however, very slight and such an observation would not necessarily explain the fact that a larger proportion of the intake was retained, as well as a larger absolute amount.

Adults.—For the adults the figures are nothing like so interesting. It was necessary to arrange a diet that should approach a minimum at which equilibrium might be expected for each of the three elements, calcium, phosphorus, and nitrogen, in order to detect slight variations in diet, and at the same time include some of all the foods consumed by the children, since the experiments were carried out simultaneously. The 133 gm. of milk used daily furnished 50 per cent of the calcium, 20 per cent of the phosphorus, and 10 per cent of the nitrogen. It is apparent that only for calcium should significance be attached to the results. On the evaporated milk the calcium balances were -0.21, -0.08, -0.02, 0.06 gm. per day; while the corresponding figures for pasteurized milk were -0.09, -0.16, -0.01, -0.03. Half of these balances are slightly in favor of evaporated and half of pasteurized milk. Therefore, the two forms of milk proved equally satisfactory as sources of calcium for adults.

SUMMARY.

A study was made of the comparative influence of evaporated and commercially pasteurized milk on the calcium, phosphorus, and nitrogen metabolism of four children, 3, 4, 8, and 12 years old, and of three adults.

All the balances for the children were positive and ranged for

calcium from 0.11 to 0.64 gm. per day; for phosphorus from 0.11 to 0.33; for nitrogen from 1.18 to 2.21.

For the children, the diet with evaporated milk resulted in a higher phosphorus retention in all four cases and a higher nitrogen and calcium retention in three of the four.

For the adults only the calcium balances are considered significant. Half of these are in favor of evaporated and half in favor of pasteurized milk.

The evaporated milk, therefore, appeared to be a satisfactory source of calcium, phosphorus, and nitrogen, slightly superior to the pasteurized milk.

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THE DISTRIBUTION AND RECOVERY OF GLUCOSE INJECTED INTO ANIMALS.

By OTTO FOLIN, HARRY C. TRIMBLE, AND LLOYD H. NEWMAN.[*]

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

In connection with their discussion of the nature of the glucose threshold in 1922, Folin and Berglund expressed (1) the thought that tissues may be able to take up and hold as high concentrations of sugar as is circulating in the blood and that therefore the distribution of the free absorbed sugar rather than glycogen formation in the liver is the first factor to come into play to prevent excessive accumulation of sugar in the blood. They also advanced the, probably erroneous, hypothesis that the different effects of sugars other than glucose on the blood sugar level might be due to the absence of these other sugars in the tissues. Throughout their paper they take for granted that absorbed sugar remains within the body as sugar or as glycogen until it is used up. The correctness of this fundamental concept has been more or less seriously questioned. In fact when the investigations of which this paper is a part were commenced, more than 3 years ago, the idea had become fairly generally accepted, in this country, that it is not possible to demonstrate experimentally that the concept is a true one. These doubts originated with the observations of a number of early workers[1] that glucose injected into animals cannot be recovered analytically either as sugar or as glycogen. The difficulties experienced by Kleiner and Meltzer (2) and by Palmer

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[*] National Research Council Fellow in Medicine, 1923–25.

[1] Since the papers of Kleiner (2) and of Palmer (3) contain very extensive references to the earlier literature, we have not considered that space should be taken to repeat these summaries here. In addition to these and also other recent papers mentioned in the text attention should be directed also to an article by Bissinger and Lesser (4) who have devoted some attention to this problem in connection with their investigation of the action of insulin.

(3), who studied the question in greater detail, in attempting to account quantitatively for injected or ingested sugar on the basis of regional analyses served to emphasize the doubt.   Macleod (5) summarizing the situation at the beginning of the year 1926, says: "It has been found by several workers that it is impossible, by analysis of the entire animal, to account for more than a fraction of the amount of glucose injected into it shortly before death."

Our research began with the conviction that it should be possible to account analytically for glucose injected into an animal's body.   After a very extensive series of experiments on dogs we were forced to admit to ourselves, however, that our recoveries were not much superior to those which had been obtained by others.

In computing the total sugar recovered in these experiments we relied upon analyses of those tissues (muscle, liver, and blood) which at that time appeared to us to be of the greatest importance in sugar disposal.   A careful consideration at that time raised the possibility that significant deposits of sugar might have been overlooked in our survey.   So, still retaining the conviction that sugar placed within an animal's body must remain there for at least a brief period we undertook in the spring of 1925 a series of experiments with small animals which could be analyzed *in toto* for sugar.   At that time we were able to obtain a satisfactory accounting for the injected sugar as we will bring out a little farther along in this paper.   Because of some interruptions due to interest in other features of carbohydrate metabolism we have delayed our report.   Recently Cori and Cori (6) have published some interesting papers in which they show on the basis of 4 hour metabolism experiments with rats that 90 per cent of the absorbed sugar is either burned, or stored as glycogen.   90 per cent must be considered as well within reasonable limits of error in such experiments.

It seems to us, however, that metabolism experiments of the sort described by Cori and Cori do not supply an entirely satisfactory answer to the fundamental problem.   No one can really doubt that absorbed carbohydrates ultimately yield CO_2 and H_2O in proportion corresponding to carbohydrates, but such ultimate complete oxidation does not explain why investigators have failed to recover added sugar when the animal which has received the

sugar is killed before there has been time to oxidize much of it and when as a matter of fact little or none of it can be found in the form of extra, stored, glycogen.

Within 5 minutes after injecting 2 gm. of glucose per kilo of body weight into the circulation of dogs, all but from 15 to 30 per cent has left the blood and only a small part of the remaining 70 to 85 per cent has been traced into the tissues. In the course of analyses of different tissues in such experiments one finds that the sugar concentration in muscles is far below that of the blood, nor does one find a common level of the sugar in blood and muscle at any later period.

In Table I we give the results obtained in four typical experiments in which the sugar was determined 30 minutes after the

TABLE I.

Sugar Concentration in Blood and Muscle before and 30 Minutes after Injection of 3 Gm. of Glucose per Kilo of Body Weight of Nephrectomized Dogs.

Experiment No.	Sugar in mg. per cent.			
	Before injection.		After injection.	
	Blood.	Muscle.	Blood.	Muscle.
1	62	34	500	110
2	95	49	380	136
3	78	72	412	154
4	90	49	286	141

injection. We have many others obtained under somewhat different conditions, but as these give essentially the same picture and do not differ materially from similar results obtained by others we refrain from reproducing them. It is clear from such results that the plausible postulate of a common sugar level for the blood and for such general tissues as muscles does not correspond to the facts.

In view of the very great difference between the sugar concentration in the blood and in muscles illustrated in Table I it is not possible to assume that there can be free and unregulated diffusion of glucose between these two localities. Nor can one fall back on the glycogen formation as an adequate explanation of the

low sugar levels in the muscles.   If the speed of the glycogen-forming process were so great as to account for the large difference in sugar concentration in muscles and blood, it would have to be an extremely rapid process, so rapid that the sugar concentration in the blood would have to sink to approximately normal levels in the course of a few minutes.   This point needs emphasis because many recent observations, notably those of Hagedorn (7), have shown that during periods of sugar distribution the arterial blood does give off much sugar to the tissues.   Differences up to 30 mg. per cent between the sugar content of arterial and venous blood

TABLE II.

Comparison of Glycogen Content before and after Intravenous Injection of Glucose.

Kidneys removed.   First samples of tissue taken.   Glucose injected (3 gm. per kilo).   Second group of tissues removed 30 minutes after the injection.   Glycogen is recorded as glucose.

Experiment No.	Muscle.			Liver.			Blood sugar when second set of tissues was taken.
	Before.	After.	Increase.	Before.	After.	Increase.	
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	1085	1147	62	571	872	301	752
2	1042	1108	66	2830	2976	146	328
3	950	994	44	3864	4111	247	
4	1245	1306	61				696

In Experiments 1 and 2 glycogen was determined by the Pflüger method.   In Experiments 3 and 4 it was measured by determining the increase in total sugar after hydrolysis of the tissues by 0.5 N acid.

were repeatedly found by Hagedorn.   At least under our experimental conditions the glycogen formation is, as a matter of fact, so slow that 30 minutes after a sugar injection it is barely possible to be sure that the glycogen content of the muscles actually has increased.   This fact is demonstrated by the glycogen determinations recorded in Table II.   It is possible, of course, that the glycogen formation was abnormally slow in our animals because of the operative procedures and the use of amytal anesthesia.   As this point is immaterial to our problem, the analytical recovery of injected sugar, we have not attempted to elucidate it experimen-

tally. So far as concerns loss of sugar from arterial blood it may be pointed out that all the sugar leaving the arterial blood has not necessarily gone into muscle tissue only.

The large losses of glucose encountered in our early experiments with dogs might signify to be sure a rapid transformation of injected sugar into non-carbohydrate material, but they certainly could not be accepted as proving the point until the facts had been verified by analyses actually representing the *entire* animal. For such supplementary investigations it was impractical to use as large animals as dogs since an indispensable feature must be the transformation of the whole animal into one homogeneous mixture.

We have usually employed guinea pigs. A uniform hash can easily be prepared from a freshly killed guinea pig by first dipping it in boiling water for a moment to remove the hair and then passing it through an ordinary meat grinding machine.

For this kind of work it was first of all necessary to determine whether it is possible to recover a sugar when it had been added directly to the hash. Since sucrose is not destroyed within the animal body we first made the required check work with this sugar.

The analytical results representing the recovery of sucrose added directly to hashed guinea pigs are given in Table III. The recovery of the added sugar is certainly excellent, the largest error encountered being only 5 per cent.

The next step was to introduce the cane sugar into the jugular veins of the living guinea pigs (under amytal anesthesia). Half an hour after the sugar injection the animals were killed by a blow on the head. The dead animals were then freed from hair, hashed, and analyzed exactly as in the series represented in Table III.

Since the experiments in our hands have given perfectly satisfactory recoveries of injected sugar it seems desirable to describe the work in some detail.

Guinea pigs weighing 400 to 500 gm. were used. After 24 hours fasting the pigs were anesthetized by an intraperitoneal injection of 50 mg. of amytal. Both kidneys were then removed, and after closing the incision 12 cc. of a 10 per cent solution of sucrose were slowly injected by means of a Luer syringe into the jugular vein. The animals were then left in a warm place for 30 minutes, after

which they were killed by a blow on the head and plunged into hot water to loosen the hair.

After the hair had been removed each entire animal was passed through a meat chopper until finely hashed.   To the hash in a beaker were then added about 500 cc. of hot distilled water and the mixture was heated in boiling water with constant stirring for 10 minutes.   After being allowed to settle, the supernatant liquid was removed by decantation and replaced by fresh hot water (200 cc.), and the heating with stirring, settling, and decantation was repeated.   In all four extractions were thus made with a total of about 1200 cc. of hot water.   The combined extracts and residues were thoroughly mixed, cooled, and the volume was measured.

To 60 cc. of the turbid mixture were then added 20 cc. of 10 per cent sodium tungstate and 20 cc. of 2/3 N sulfuric acid (with

TABLE III.
Recovery of Sucrose after Addition to Hashed Guinea Pigs.

Experiment No.	Sugar added to tissue.	Sugar recovered from extracts.
	mg.	*mg.*
1	1010	998
2	1200	1228
3	1200	1224
4	1200	1239
5	1200	1156

Average recovery when 1200 mg. of sucrose were added = 1211 mg.

shaking).   This protein precipitation was followed by filtration and in the filtrate the reducing sugar was determined before and after hydrolysis of the cane sugar.   The difference should represent the sucrose.

For the determination of the free reducing sugar 15 cc. of the extract were acidified by the addition of 5 cc. of 0.1 N sulfuric acid and were then shaken with 1.5 gm. of Lloyd's alkaloidal reagent. The final filtrate then obtained usually showed a suitable concentration so that 2 cc. could be used for the Folin-Wu sugar determination (8).

For the total sugar determination 10 cc. of the protein-free extract were mixed with 10 cc. of N sulfuric acid and the acidified mixture was allowed to stand at room temperature for 24 hours.

This mild treatment is sufficient for the hydrolysis of the cane sugar and does not affect glycogen. The solution was then carefully neutralized with solid sodium carbonate. To 15 cc. of the neutralized solution were then added 5 cc. of 0.1 N acid and the mixture was shaken with 1.5 gm. of Lloyd's reagent and filtered, just as in the preformed sugar determinations, and the total sugar was determined by the Folin-Wu process.

In connection with these determinations it should be mentioned that invert sugar gives only 95 per cent as much cuprous oxide as an equal weight of glucose (9). Since cane sugar gives 105 per cent of invert sugar these two factors cancel each other.

TABLE IV.

Recovery of Sucrose ½ Hour after Intravenous Injection into Guinea Pigs.

Experiment No.	Total reducing substance after hydrolysis. (A)	Free sugar before hydrolysis. (B)	Sucrose recovered. (A)–(B)	Sucrose injected.
	mg.	*mg.*	*mg.*	*mg.*
1	1482	408	1074	1200
2	1773	533	1240	1200
3	1678	642	1036	1200
4	2105	809	1256	1200
5	1702	618	1084	1200
6	1511	396	1115	1200
7	1888	627	1261	1200
8	1605	554	1051	1200

Average recovery = 1139 mg., or 94 per cent.

From the figures recorded in Table IV it may be seen that the recovery of cane sugar injected into living guinea pigs was very nearly as quantitative as the recoveries obtained when the sugar was added to the previously killed and minced animals. The recovery of the injected cane sugar seems, if anything, a little too good, because cane sugar is so easily inverted and in the course of half an hour's circulation some of the sugar might have become accessible to the invertases in the pancreas and the digestive tract.

The cane sugar experiments of Tables III and IV have, of course, no direct bearing on the problem of glucose recoveries, but they show that added or injected sugar, in so far as it is still present at the end of an experiment, can be determined. To ourselves this

fact proved distinctly helpful, because earlier, when working with dogs, we had made use of the same principle and had found that when cane sugar was injected into dogs we were not able to account for the sugar by means of the regional analyses made.   The losses encountered with cane sugar were then in fact almost as large as the corresponding losses encountered with glucose.   In the light of the cane sugar recoveries reported in Tables III and IV these earlier losses of sucrose serve only to emphasize the fact that quantitative recovery of an injected substance by means of regional tissue analyses is very difficult of attainment.

The recovery of glucose added to a freshly prepared guinea pig hash or injected into the living animal is necessarily a different proposition from that of recovering a foreign sugar like sucrose. Deduction of the preexisting glucose in the animal is impossible except by the rather uncertain process of control experiments which involve the assumption that one guinea pig contains the same amount of preformed sugar as another.   Weak and faulty as this assumption is we have nevertheless become convinced that by its help it is possible to settle better than in any other way the question as to whether injected glucose is really transformed into non-carbohydrate material so rapidly as to account for the sudden "disappearance" of about one-half of the injected material. Errors in the above mentioned assumption cannot all be on the same side and it is therefore only a matter of the number of experiments made until the average result will be substantially correct. As a matter of experience, however, the variations encountered are not large in similarly treated fasting guinea pigs.

It may be pointed out in passing that the use of control animals as a means of estimating the original carbohydrate content of entire small animals is after all in principle and application not materially different from the control determinations which must be made of the different tissues when working with large animals. While we have made many control determinations in different lobes of the liver and in corresponding left and right muscles and have found quite satisfactory agreements for their sugar and glycogen content, the fact remains that such control determinations of sample tissues may not truly represent the whole of that tissue in the body, especially in the case of the muscles.

For the glucose experiments recorded below we selected a lot of

guinea pigs about the same age and size and believed to be of the same stock.   Three of the animals were anesthetized and nephrectomized as in the cane sugar experiments and 1200 mg. of glucose were slowly injected into the jugular vein of each.   Three control animals received the same treatment except for the glucose injection.   After ½ hour the animals were killed, hashed, and extracted as were the cane sugar animals.   The extracts from each set of three animals were combined and analyzed for free sugar and for hydrolyzable carbohydrates (glycogen).   This experiment was repeated with a second lot of six guinea pigs.   The total amount of glucose injected in each set of three guinea pigs was 3600 mg.   In the first set, we found 5516 mg. of free sugar in the injected animals and 1585 mg. in the controls.   Subtracting 1585 from 5516 we got 3931 mg. of glucose or 331 mg. more than we had injected.   For the total carbohydrate (glucose + glycogen) we obtained 9467 mg. and 5856 mg.   Subtracting from these figures those for the free sugar, 5516 and 1585, there remain 3951 mg. for the glycogen of the injected animals and 4271 mg. for the controls. These glycogen figures seem to us very instructive.   The injected animals gave us actually 320 mg. less of glycogen than were found in the controls.   The figures, therefore, clearly indicate that practically no glycogen had been formed from the injected sugar, a finding which is quite in harmony with our recovery of 9 per cent more glucose than had been injected.   Here we have, therefore, not even a suggestion of any loss of injected glucose.

From the second set of three guinea pigs which had received 3600 mg. of glucose we obtained 5071 mg. of free sugar, while the three controls yielded 1264 mg.   Here we have again recovered rather more glucose than was given, 5071—1264 or 3807 mg., instead of 3600.   The total sugar found after hydrolysis was in this case 9533 and 5758 mg., respectively, thus giving us 4462 and 4494 mg. of glycogen, or 32 mg. less in the injected animals than in the controls.

The recoveries of glucose injected into guinea pigs reported in the preceding two paragraphs have convinced us that the losses incurred when sugar is injected into larger animals must be due to inadequate analytical research and not to any unknown transformation of sugar into non-carbohydrate material.

Two additional experiments of the same kind as those on guinea

pigs were made with puppies which we happened to have in the department.   These experiments are less complete, inasmuch as the hydrolyzable sugar determinations were omitted in one case and accidentally lost in the other.   The results reported in Table V show that 79 and 75 per cent of the injected glucose were recovered as free sugar.

If there were any tangible reason for believing that injected sugar might be transformed into non-carbohydrate materials, we should have continued the work with small animals.   But in the absence of any such reason it seemed more profitable to return to regional tissue analysis on larger animals in the hope of being able to explain, at least in some measure, why attempts to account for the injected sugar by means of such experiments have resulted in such unaccountably large losses.   The outcome of the guinea

TABLE V.

Recovery of Glucose ½ Hour after Intravenous Injection into Puppies.

	Experiment 1.		Experiment 2.	
	Weight.	Free sugar.	Weight.	Free sugar.
	gm.	*mg.*	*gm.*	*mg.*
Pup A (injected)......	1745	7752	1335	6857
" B (control).......	1720	3807	1320	3824
Sugar recovered.......		3945		3030
" injected........		5000		4000
Per cent recovery.....		79		75

pig experiments clearly suggested that the distribution of the injected sugar must be the responsible factor and that it does not conform to expectations; some tissues must take up more sugar than others, and certainly more than the skeletal muscles.

This conjecture has proved correct.   One important outlet for the sugar as it leaves the blood is the skin.   The skin is not a storage place for glucose or glycogen, for in a fasting condition, it contains practically no glycogen and only a trifle more sugar (about 10 mg. per cent) than skeletal muscle.   Our average sugar content for twenty-five fasting dogs is as follows: blood 84 mg. per cent, muscle 54 mg. per cent, skin 67 mg. per cent.   But as a temporary receptacle for sugar in response to sugar injections the importance of the skin seems to us to be very much greater than has been

appreciated heretofore. Our analytical figures on this point are recorded in Table VI. The dogs used in these experiments were under amytal anesthesia and the kidneys were removed before the injection of the glucose.

The figures of Table VI show that there is a vast difference between skin and skeletal muscle in relation to their response to sugar injected into the blood. The high sugar levels found in the skin are not alone sufficient to explain the large sugar deficits which we encountered in our earlier analyses. But similar though

TABLE VI.

Showing That Glucose Injections Produce Almost As High Concentrations of Sugar in Skin as in the Blood.

Mg. per cent glucose 30 min. after injection of 3 gm. per kilo.

Experiment No.	Blood.	Skin.	Muscle.
1	548	550	
2	560	576	
3	440	473	
4	640	350	
5	532	295	
6	444	291	
7	412	390	154
8	286	322	141
9	360	274	89
10	440	349	109
11	464	401	99
12	564	364	103
13	572	377	114
14	389	265	111

less pronounced accumulations of sugar are found in many other places. In fact all the glandular organs as well as the intestine and the stomach take much more sugar than do the skeletal muscles (see Table X).

The relatively large influx of sugar into the skin in response to sugar injections does not imply that the skin possesses any special capacity for holding and conserving carbohydrates. The late Dr. Bell, of this department, found several years ago that the skin under certain conditions can take up large quantities of sodium chloride; and other (unpublished) experiments made in this

laboratory have shown that other diffusible substances, as for example uric acid, also migrate into the skin in response to high levels in the blood. So far as concerns the influx of sugar into the skin, we have a number of experiments which seem to indicate clearly that the influx is the result of passive diffusion rather than of any special absorption or metabolic process.

(*A*). While skin, like every other tissue, contains some glycogen, as was shown long ago by Cramer (10), the glycogen formation in

TABLE VII.

Comparison of Sugar Concentration of Simultaneously Obtained Samples of Blood and of Skin.

Amytal anesthesia. Kidneys removed. Sugar injected, 3 gm. per kilo.

Sugar injected.	Glucose.				Levulose.	
Interval after injection.	Sugar concentration per 100 parts.					
	Experiment 1.		Experiment 2.		Experiment 3.	
	Blood.	Skin.	Blood.	Skin.	Blood.	Skin.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5			744	417		
10	668	331			640	323
20				378		
30	500	439			452	330
45			444	311		
60	332	226	364	267	205	212
90	254	182	216	228	154	136
120	190				143	107
150	143	112			100	116
180	100	64			100	109

the skin is not promoted to an appreciable extent by a single glucose injection. In one such injection experiment we found by Pflüger's method 17 mg. per cent of glycogen (before the injection) and 2 hours later, 16 mg. per cent.

(*B*). The two glucose and one fructose experiments recorded in Table VII give probably a tolerably correct picture of how the skin functions in relation to temporary hyperglycemias produced by means of sugar injections. As the blood sugar level is rapidly raised to 600 or 700 mg. per cent, there occurs at once a rapid dis-

tribution of sugar into the skin and the concentration in the latter rises to 300 or 400 mg. per cent, sometimes even to over 500 mg. per cent. Then as the blood sugar level falls below the level attained in the skin, the reverse process sets in and the skin sugar diffuses back into the blood stream. This diffusion of sugar back and forth between blood and skin conforms to what one might reasonably have assumed to take place in all the tissues of the body. The remarkable point is that the same process occurs, only to a much more limited extent, in those tissues which have the greatest need of carbohydrate food and which also possess a much greater power of converting the sugar into glycogen, namely the muscles.

It is a fact, of course, that under the influence of high blood sugar levels there is a considerable influx of sugar into the skeletal muscle, but it is also certain that the migration of the sugar into such tissues stops at such low levels that one can scarcely speak of the process as being even qualitatively similar to what takes place in the skin. Except for the sugar which doubtless gets into the lymph, the laws of diffusion and osmosis do not seem to be the determining factors in the case of the muscles.

The glycogen-forming capacity of the muscles should theoretically serve to promote the transfer of sugar into these tissues and it probably does. But this only serves to emphasize the remarkable fact that 30 minutes after an intravenous injection of glucose the amount taken by muscles is so small that one can barely be sure that the glycogen formation has gained on the glycogen destruction (see Table II).

In nephrectomized dogs injections of 3 gm. of glucose per kilo will usually yield blood sugar levels of at least 600 to 700 mg. per cent, and 3 to 4 hours must usually elapse before the blood sugar has come down to approximately normal levels. In the course of such longer experiments much of the injected sugar (or its equivalent) is of course oxidized, but one can expect that the glycogen formation continues at least so long as the sugar level in the blood remains high. Under such conditions which correspond to a certain extent with the conditions recently described by Cori and Cori (6), one finds no difficulty in showing that much of the injected glucose has been deposited in the tissues in the form of glycogen. And under these conditions the accumulation of glyco-

gen in the muscle is almost of the same order as that found in the liver. Analytical data obtained from a number of such experiments are recorded in Table VIII.

In Table IX are given, in gm., the amounts of deposited glycogen accompanying the disappearance of the injected glucose, for the first five experiments in which the circulating sugar had returned to substantially normal levels. The total weight of muscle was taken at three-sevenths of the body weight for the calculations of the muscle glycogen.

From these figures it is evident that by far the greater part of

TABLE VIII.

Glycogen Content of Tissues before and Some Hours after Glucose Injections.

Kidneys removed. Glucose injected, 3 gm. per kilo. Glycogen as mg. of glucose per 100 gm. of tissue.

Experiment No.	Muscle.			Liver.			Blood sugar at finish.	Period between injection and removal of second group of tissues.
	Before.	After.	In-crease.	Before.	After.	In-crease.		
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg. per 100 cc.*	*hrs.*
1	641	962	321	1486	2181	695	91	3
2	839	1184	345	2088	2452	364	86	3¾
3	652	1063	411	3869	4506	819	110	2¼
4	895	1160	265	1430	2509	1079	91	3⅔
5	840	1048	208	847	1521	674	108	4⅔
6	1182	1344	162	3038	4186	1148	210	4
7	715	1004	289	2353	2452	101	194	2⅔
8	554	845	291	727	1857	1130	167	3

the stored glycogen has been deposited in the muscles. The total increase in stored glycogen in muscles and liver is equivalent to more than 50 per cent of the injected glucose. In other words, without making any allowance at all for the oxidation of carbohydrates for the whole experimental period, 3 to 4 hours, we have recovered in the form of muscle and liver glycogen alone, fully as much of the injected glucose as has been obtained by regional tissue analyses begun 30 minutes, or less, after the sugar injections. If it were permissible in connection with the experiments recorded in Table IX to substitute for Cori's (6) actual determinations of oxidized carbohydrate suitable calculations for the amounts of

carbohydrate oxidized by our dogs, our analytical recoveries would probably account fairly well for all the injected glucose.

From an analytical standpoint there is one important difference between 4 hour experiments and 30 minute experiments.   In the former the unequal and constantly changing distribution of free sugar among the different tissues has run its course and the distribution has again become substantially as it was before the injection.   The injected sugar, in so far as it has not been oxidized, is stored as glycogen and under these conditions the major part is found just where one would expect to find it, namely in the muscles and the liver.   The two outstanding facts are, first, that the glycogen formation in response to intravenous glucose injections is very slow and, second, that the distribution of the injected free sugar

TABLE IX.

Comparison of Glycogen Stored in Muscle and Liver after Intravenous Injections of Glucose into Dogs.

Amytal anesthesia. Glucose injected, 3 gm. per kilo. Glycogen recorded as glucose.

Experiment No.	Stored in muscle.	Stored in liver.	Length of experiment.	Blood sugar at finish.	Weight of dog.
	gm.	*gm.*	*hrs.*	*mg. per 100 cc.*	*kg.*
1	18.5	3.0	$3\frac{1}{6}$	91	14.5
2	20.7	1.6	$3\frac{3}{4}$	86	14
3	21.7	3.7	$2\frac{3}{4}$	110	12
4	15.5	4.6	$3\frac{5}{8}$	91	13.7
5	15.9	3.3	$4\frac{2}{3}$	108	18.0

is entirely different from the subsequent distribution of the glycogen.

The unexpected character of the preliminary distribution of injected sugar, involving insignificant glucose concentrations and distinctly slow glycogen formation in the *muscles*, represents probably the main reasons why much of the injected sugar seemed to disappear and why the belief in some unknown transformation of glucose into non-carbohydrate material came to be so readily accepted.   The low sugar content in muscle is indeed remarkable.   In this connection it seems necessary to state that the values for free sugar in tissues recorded in this paper are not interpreted by us as being even approximately correct.   Through other work

done in this laboratory during the past year we know that the absolute sugar values are very much lower than the figures obtained by the Folin-Wu method. That aspect of the sugar problem is relatively unimportant in this research, however, because the Folin-Wu method does give consistent values and does give the added sugar which results from sugar injections, and it is such added glucose that we have tried to follow in this research.

Notes upon Materials and Methods.

Sugars.—C.P. glucose and sucrose were employed. The purity of each lot was checked by analysis. Anhydrous glucose prepared by the United States Bureau of Standards was used in preparing the standards for the Folin-Wu procedure. Freshly prepared solutions were used in each experiment.

Extraction and Determination of Free Sugar in Individual Tissues.—In our determination of sugar concentrations in individual tissues our procedure was as follows:

The tissue was rapidly removed from the animal body, and all possible blood was pressed out. It was weighed to gm. only on a small torsion balance and thrust at once into several times its weight of boiling water contained in a beaker, which was supported over a direct flame. Vigorous heat was maintained all of the time and shortly after the introduction of the tissue the liquid again boiled steadily.

Then the tissue was cut into small sections in the boiling water by means of scissors or was hashed by passage through a meat chopper. Then the beaker and its contents were transferred to a boiling water bath and left there until further treatment was possible. As soon as convenient the tissue was ground in a mortar to disintegrate it thoroughly and returned to the extracting fluid. After 15 minutes the liquid was decanted off and replaced by a volume of boiling water approximately equal to the weight of the tissue. After heating for 10 to 15 minutes the liquid was again decanted and a third and a fourth extraction made in a similar way. The extracts were combined, cooled, thoroughly mixed, and the total volume determined.

An aliquot portion, usually 20 cc., was treated with tungstic acid and Lloyd's reagent as previously outlined in the text (see experiments on recovery of sucrose from the guinea pig), and then the sugar was determined by the Folin-Wu method.

Glycogen.—The Pflüger (11) procedure was employed with the modification that the glucose produced by hydrolysis was determined by the Folin-Wu blood sugar method. In bringing the hydrolysates to volume for analysis they were diluted to such an extent that the inorganic salt content did not interfere with development of color with the Folin-Wu reagent.

In some cases it was desired to know both the free sugar and the glycogen content of tissues. This was accomplished by removing an aliquot portion

of the extract for determination of the free sugar. Then to the remainder of the fluid extract and the tissues sufficient sulfuric acid was added to make the final concentration 0.5 N. This mixture was heated on the steam bath for 6 hours. Then an aliquot portion was withdrawn, neutralized, treated with tungstic acid and Lloyd's reagent, as described in detail in the text (see sucrose experiments), and then the total reducing substance was measured. The difference between the total reducing substance after hydrolysis and the free sugar was taken to be glycogen. As it is realized that this procedure is not so accurate as that of Pflüger it was employed in but few of the experiments and these are clearly indicated in the text.

Anesthesia.—Amytal (isoamylethylbarbituric acid) prepared for administration as described by Page (12) was employed where anesthesia was necessary. To small animals it was administered intraperitoneally in doses of 5 mg. per 100 gm. of body weight. With dogs it was found preferable to inject it intravenously, since in this way it is possible to induce anesthesia in a very short interval (1 or 2 minutes) without any struggling on the part of the animal. This is particularly advantageous in experiments in which it is desired to make determinations of muscle glycogen.

Our experience with this anesthetic has been similar to that of those who have reported that it leaves the blood sugar at very nearly the normal level throughout a variety of operative procedures. Whether such observations signify that sugar metabolism is entirely normal under this anesthetic is open to question. Hines, Boyd, and Leese (13) found that dogs receiving glucose intravenously at a rate of 4 gm. per kilo per hour excreted more of it while under the influence of the anesthetic than the same animals did in experiments without anesthesia. Also the blood sugar rose to a higher level in the first portion of the injection period when amytal anesthesia was employed. In our experiments it will be noted (Table IX) that the length of time required to dispose of equivalent quantities of sugar varied. We are inclined to believe that variations in the depth of anesthesia may have been a factor in causing these variations. It is quite probable then that sugar metabolism may proceed at a somewhat slower rate in the amytalized animal, but we have observed nothing which would cause us to think that it is fundamentally different from that of the unanesthetized animal.

Best, Hoet, and Marks (13) have reported difficulties in attempts to study glycogen formation in animals under amytal anesthesia. They state that under the influence of amytal alone loss of muscle glycogen occurred and that doses of insulin which reduced the blood sugar to 70 mg. per cent did not prevent the loss. Such glycogen loss has been contrary to our experience when amytal alone was administered to dogs.

Sampling Tissues for Glycogen Determinations.—In those experiments in which glycogen was determined by Pflüger's method the procedure was as follows: The abdomen was opened by an incision on the midline. One of the large lobes of the liver was grasped and a ligature of heavy cord passed around it and drawn tight and tied. The cord would cut through the liver substance until the large blood vessels were reached and it could

easily enclose these so tightly that hemorrhage would not occur. A large portion of the lobe below the ligature was cut off, blood squeezed out, and rapidly weighed to gm. upon a torsion balance. Then the sample was slashed with a scissors and dropped into an equal volume of boiling 60 per cent potassium hydroxide. With further application of heat and stirring, solution quickly ensued. At the conclusion of an experiment the total weight of the liver was computed from that of the residue removed from the body at the conclusion of the experiment plus the weight of the samples withdrawn earlier.

For a comparison of muscle glycogen before and after glucose disposal experiments, corresponding muscles from the right and left rear legs were employed. Particular effort was made to obtain the entire muscle for study in order that local irregularities in glycogen distribution might be

TABLE X.

Showing That Glucose Injections Produce High Concentrations in Many Tissues.

Mg. per cent glucose 30 minutes after injection of 3 gm. per kilo into nephrectomized dogs.

Experiment No.	Blood.	Spleen.	Intestine.	Stomach.	Liver.	Remarks.
1	548		485	430		
2	560	202	326		399	
3	440	198	186			
4	380				505	Levulose, 3 gm. per kilo.
5	668				515	" 3 " " "
6	872	334	641	525	864	

avoided. The muscle was hashed, weighed to gm., and at once dissolved in an equal weight of boiling 60 per cent potassium hydroxide. In control experiments the glycogen content of corresponding muscles did not differ more than 30 mg. per 100 gm. when removed in this way and analyzed as described previously.

Distribution of Injected Glucose into Various Tissues.—In Table X we include some data from our experiments upon dogs which show that when the blood sugar level is elevated high concentrations of sugar are likewise to be found in some glandular organs and in the stomach and intestines. In animals whose blood sugar was at a normal level we have as average sugar concentrations for these tissues: blood 84, spleen 42, intestine 63, liver 254 mg. per cent. Because of the rapidity with which liver glycogen is converted into glucose, we believe that these and other published values of free sugar in the liver do not truly represent the situation there.

We wish to express our thanks to those who have given us assistance with our experimental work from time to time; especially are we indebted to Mr. C. A. Morrell and to Miss Rachel M. Smith.

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THE VOLUMETRIC ESTIMATION OF ALKOXYL GROUPS IN ORGANIC COMPOUNDS. A MODIFICATION OF THE ZEISEL PROCEDURE APPLICABLE TO METHOXYL-, ETHOXYL-, AND SULFUR-CONTAINING COMPOUNDS.

By EMILY P. EATON and EDWARD S. WEST.

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis.)

The original method of Zeisel for the determination of alkoxyl groups (1) has undergone several modifications, especially with respect to apparatus used and in procedures to make it applicable to sulfur-containing compounds (2). Kirpal and Bühn (3) introduced a modification which can be applied to methoxyl compounds containing sulfur. They digest the substance to be analyzed with hydriodic acid in the usual way and absorb the resulting methyl iodide in pyridine, thereby forming pyridinium methyl iodide. After evaporation of the pyridine and removal of any hydrogen sulfide present as a result of the reduction of sulfur by hydriodic acid, the pyridinium methyl iodide is dissolved in water and the iodine titrated with standard silver nitrate with sodium chromate as an indicator. Hewitt and Jones (4) pointed out that evaporation of the pyridine is unnecessary in the absence of hydrogen sulfide and that the iodine may be quickly determined by dilution of the pyridine solution with water, acidification with nitric acid, and titration by the method of Volhard. According to Kirpal and Bühn (5) the method can be used only with methoxyl compounds, ethyl iodide being incompletely absorbed by pyridine even at 75°C. This necessitates the use of some such agent as cadmium sulfate (6) to remove hydrogen sulfide from the ethyl iodide vapors when ethoxyl compounds containing sulfur are being analyzed.

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The writers have found that the iodine of pyridinium alkyl iodides is quantitatively liberated by oxidation with potassium iodate in acid solution. The observation has been made also that ethyl iodide is completely absorbed by pyridine at 80°C. if the proper absorption apparatus be used. Based upon these facts the

B
CONDENSER

b

c

D

a

C

PYRIDINE ABSORPTION
TUBE

CO₂
INLET

A
DECOMPOSITION
FLASK
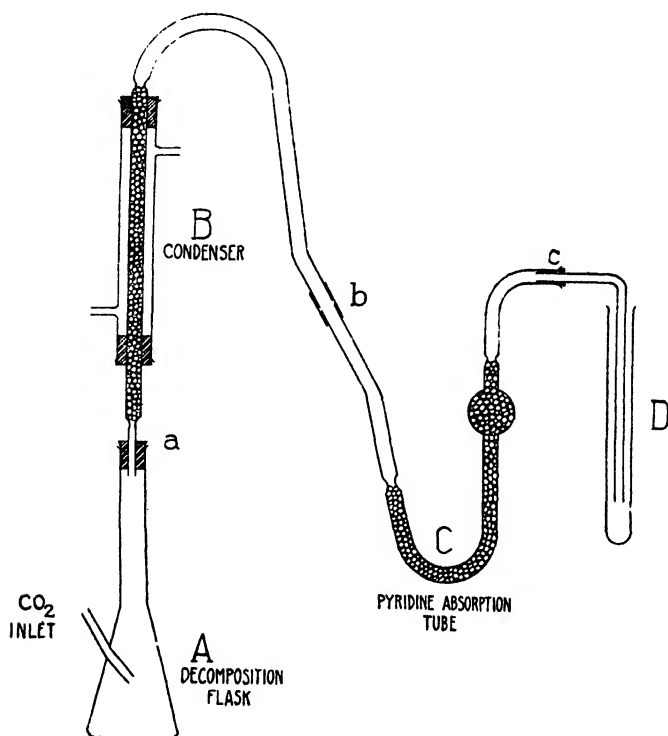
Fig. 1."

following modification of the Zeisel procedure is suggested as being applicable to both methoxyl and ethoxyl compounds including those which contain sulfur.

Apparatus.

The apparatus is set up as shown in Fig. 1. It consists essentially of three parts; a decomposition flask, A, (100 cc.), a water-cooled reflux tower, B, and a U absorption tube, C. The latter is

conveniently made from Pyrex tubing of 10 mm. inside diameter and long enough to contain the requisite quantities of beads and pyridine. The same tubing may be used to advantage in constructing the reflux tower. The water condenser of the tower should be 8 to 10 inches long. When properly made one absorption tube containing 5 cc. of pyridine and heated to 80°C. is sufficient for the complete absorption of the ethyl iodide from an ordinary sample. The connections at *a*, *b*, and *c* should be of good rubber and tight, with the ends of the glass tubes brought as close together as possible in order to minimize the contact of rubber with the alkyl halides. Alcoholic silver nitrate may be placed in *D* to indicate whether absorption is complete. Ordinarily it is unnecessary.

Reagents.

Hydriodic Acid.—A constant boiling solution prepared according to Houben-Weyl (7), also a product supplied by Merck.

Red Phosphorus.—Prepared according to Houben-Weyl (8).

Pyridine.—Mallinckrodt's c.p. grade.

Carbon Dioxide.—The product of a Kipp generator bubbled through dilute silver nitrate solution.

Procedure.

An accurately weighed quantity (0.2 to 0.3 gm.) of the compound to be analyzed is placed in the flask *A* with 15 cc. of hydriodic acid, 0.5 gm. of red phosphorus, and some boiling stones. 5 cc. of pyridine are put into the absorption tube, *C*, and the apparatus connected as shown in Fig. 1. A slow stream of carbon dioxide (one bubble every 2 to 3 seconds) is admitted through the side tube of flask *A* which is then heated to 130–135°C. in a glycerol bath. The absorption tube, *C*, is heated to approximately 80°C. in a water bath when ethoxyl compounds are being analyzed. It is unnecessary to heat *C* in the analysis of methoxyl compounds. Ordinarily decomposition and absorption are complete in an hour. Some compounds may require longer.

The pyridine solution is removed from the absorption tube to a beaker with thorough rinsing and then transferred to the distilling flask (500 cc.) of an apparatus suitable for distilling halogens (ground glass connections). The solution is diluted to approxi-

mately 250 cc. with water, acidified with 50 per cent sulfuric acid, potassium iodate (0.5 gm.), and boiling stones added, and the liberated iodine distilled into 200 cc. of 5 per cent potassium iodide and titrated with 0.1 N thiosulfate in the usual manner. If the compound contains sulfur the pyridine solution from the absorption tube is diluted to 500 to 600 cc. with water and boiled until all odor of pyridine and hydrogen sulfide has disappeared. It is then subjected to oxidation, distillation, and titration as outlined. The essential reactions involved may be represented by the equations:

$$5(C_5H_5NR) I + 5H_2SO_4 = 5HI + 5(C_5H_5NR)HSO_4$$
$$KIO_3 + H_2SO_4 = HIO_3 + KHSO_4$$
$$5HI + HIO_3 = 3I_2 + 3H_2O$$
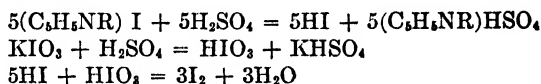
It will be noted that five-sixths of the iodine titrated comes from the pyridinium alkyl iodide and one-sixth from the iodate used. Accordingly five-sixths of the titration value represents alkoxyl. 1.00 cc. of 0.1 N $Na_2S_2O_3$ = 1.00 cc. of 0.1 N I_2 = 0.0045 gm. (1 millimol) of OC_2H_5 = 0.0031 gm. (1 millimol) of OCH_3. For example, 13.13 cc. of 0.1 N $Na_2S_2O_3$ were required to titrate the iodine obtained from 0.3020 gm. of a certain ethoxyl compound. The iodine corresponding to ethoxyl then was $\frac{5}{6} \times 13.13 = 10.94$ cc. of 0.1 N. The weight of alkoxyl was $10.94 \times 0.0045 = 0.0492$ gm. and the per cent of alkoxyl, $\frac{0.0492}{0.3020} \times 100 = 16.30$.

The method was tried on three methoxyl compounds and one ethoxyl compound. To test its applicability to sulfur-containing compounds, analyses of the ethoxyl compound were made in which the stream of carbon dioxide was bubbled through a saturated aqueous solution of hydrogen sulfide before entering the digestion flask. The hydrogen sulfide was passed in for about 10 minutes at the beginning of an analysis after which pure carbon dioxide was passed as usual throughout the course of the digestion. In all cases the odor of hydrogen sulfide was very strong in the pyridine solution after the absorption of ethyl iodide was completed. Control determinations on all compounds were made by the usual Zeisel procedure in which the alkyl iodide was absorbed in alcoholic silver nitrate and the silver iodide weighed. Representative results are given in Table I.

DISCUSSION.

It will be noted that the values obtained by the pyridine oxidation method correspond favorably with those given by the Zeisel procedure. One good point of the method is that great care need

TABLE I.

Analysis by the Pyridine-Oxidation Modification.

Compound.	Without H₂S.				With H₂S.				Alkoxyl found by Zeisel procedure.
	Weight of sample.	0.1 N iodine.	Weight of alkoxyl found.	Alkoxyl.	Weight of sample.	0.1 N iodine.	Weight of alkoxyl found.	Alkoxyl.	
	gm.	*cc.*	*gm.*	*per cent*	*gm.*	*cc.*	*gm.*	*per cent*	*per cent*
Tetramethylglucose cycloacetoacetic acid, 41.08 per cent OCH₃ theoretical.	0.1455	18.86	0.0585	40.25					40.28
	0.0546	7.07	0.0219	40.20					40.07
Methyl salicylate, 20.39 per cent OCH₃ theoretical.	0.2800	17.23	0.0534	19.07					19.50
	0.2572	16.18	0.0502	19.50					
Vanillin, 20.39 per cent OCH₃ theoretical.	0.2673	16.85	0.0522	19.54					20.09
Glucose cycloacetoacetic acid ethyl ester, 16.41 per cent OC₂H₅ theoretical.	0.1856	6.68	0.0302	16.21	0.2756	9.78	0.0440	16.02	16.07
	0.2929	10.38	0.0467	15.94	0.2567	9.03	0.0407	15.85	15.96

not be taken to use hydriodic acid entirely free from sulfur since any hydrogen sulfide may be easily removed before oxidation with iodate. The time required for an analysis is appreciably less than that by the regular Zeisel method and also the modification of

Kirpal and Bühn, though probably more than for the modification of Hewitt and Jones. Applicability to both methoxyl and ethoxyl compounds with or without sulfur is a decided advantage. One disadvantage of the method is that it is necessary to have an apparatus for the quantitative distillation of iodine. This objection probably need not apply in the majority of laboratories.

SUMMARY.

A modification of the Zeisel method for the estimation of alkoxyl groups is proposed based upon absorption of the alkyl iodide in pyridine followed by oxidation of the pyridinium alkyl iodide with potassium iodate, distillation of the iodine, and titration with thiosulfate. When the apparatus described is used the procedure is applicable to both methoxyl and ethoxyl compounds and those which contain sulfur.

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MODIFICATIONS OF RAST'S MICRO METHOD FOR MOLECULAR WEIGHT DETERMINATIONS.

BY JAMES H. C. SMITH AND WILLIAM G. YOUNG.

(From the Coastal Laboratory of the Carnegie Institution of Washington, Carmel-by-the Sea, California.)

In the course of investigations of photosynthesis in plants it has become clearly evident that more precise knowledge of the cell constituents is a prerequisite to an understanding of the mechanisms concerned. Because of the fact that a great deal of labor and time are involved in obtaining even small quantities of many of these compounds in a high state of purity, micro methods must be considered for obtaining the desired information. This applies also to the pigments of the chloroplasts. Although considerable work has been done on these pigments, investigations in this laboratory have revealed the fact that several fundamental properties as, *e.g.* the molecular weights of such compounds as carotin and xanthophyll, have not been established with certainty and that their exact determination offers a number of difficulties. The method to be described has proved to be very satisfactory for a variety of substances and requires only very small amounts of material and simple apparatus.

Rast (1) proposed a simple micro method for the determination of molecular weights which has been applied by Houben (2), Wittka (3), Sadikov and Michailov (4), Mason (5), Pastak (6), and Carlsohn (7) to a variety of compounds. Rast's method is, in short, the adaptation of the ordinary method used by organic chemists for the determination of the melting point of organic compounds to the determination of the lowering of the melting point of camphor by a solute. From this depression the molecular weight of the solute is calculated. Camphor has a molecular lowering of 39.7° and has the advantages of ease of purification and great solvent powers. It is very fluid in the liquid state and crystallizes readily without supercooling.

The object of this communication is to describe certain modifications of the original method which further increase its usefulness and accuracy and to add data to those already existent to show the dependability of the procedure and its limitations. In Rast's method, the substance, the molecular weight of which was to be determined, was melted with a definite amount of camphor and the mixture allowed to solidify; a portion of this was then used for the determination of the melting point of the mixture. This is one of the steps in the procedure which has been changed, because thereby smaller amounts of the substance can be used
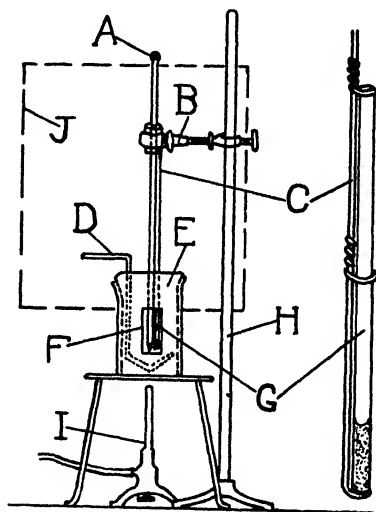
Fig. 1.

and because in some instances it was observed that Rast's procedure has decided disadvantages when waxy mixtures were obtained or readily oxidizable solutes were being investigated (carotin or xanthophyll). The present procedure also obviates any possible loss of camphor through sublimation.

Apparatus.—The apparatus which was finally found to be the most useful in our laboratory is diagrammed in Fig. 1. A tall 300 cc. beaker (*E*) was covered with an asbestos sheath to eliminate the large heat loss and almost filled with Parawax or glycerol. Two windows (*F*), 2.5 × 4.5 cm. were cut in the asbestos cover-

ing so that the melting point tube (*G*) could easily be seen by the illumination from the electric light placed directly behind the bath. The melting point tube was held *completely submerged* by the special holder (*C*), a frame made from copper wire and suspended from clamp (*B*), so that the melt in the capillary tube was close to the mercury bulb of the thermometer (*A*). The thermometer used in the earlier determinations was an ordinary grade of laboratory thermometer graduated in whole degrees. Splendid results were obtained with it but the accuracy and ease of making the temperature readings were much increased by resorting to a thermometer, graduated in 0.1° divisions, and with a working range from 100–200°C. The bath liquid was stirred by hand with a stirrer (*D*). The source of heat was a small Bunsen burner (*I*). A sheet of paper (*J*), hung just back of the bath so that its lower edge was level with the top of the rear window, served to cut off the glare from the light, and made the manipulation more accurate. A second thermometer hung so that the bulb came at the middle point of the thermometer (*A*) recorded the average temperature of the exposed stem. This average temperature was used in making the correction for the exposed stem.

Manipulation.—It is essential that the melting point tubes have very thin walls so that there will be no temperature lag between the bath and the substance in the tube. Tubes 2 mm. × 6 cm. were found to give the best results. The tubes were made by drawing out thin walled test-tubes, cutting to the desired lengths, and sealing one end with as little accumulation of glass as possible. The tubes were stored in a desiccator to minimize the amount of absorbed moisture.

When a molecular weight was to be determined, a tube was taken from the desiccator, wiped with a clean dry cloth, and weighed on a good analytical balance to the nearest 0.01 mg. by the method of swings. From 1 to 3 mg. of the substance, the molecular weight of which was to be determined, was then introduced into the tube. The tube was wiped again and reweighed with the same accuracy. Now the desired amount of camphor (about 10 times the weight of the unknown) was put on top of the sample and roughly weighed so that it was known that approximately the right amount had been added.

The open end of the tube was then sealed by heating slowly from about 1 cm. back of the opening to the open end. This drove out the adhering camphor along with some of the air. When the fumes of the camphor ceased to escape the end was melted together without difficulty. The tube was then cooled, wiped with a dry cloth, and reweighed accurately. It is a great advantage to use a closed tube, because the tube can be completely submerged, thus avoiding condensation of camphor on the cooler exposed walls and as many readings as desired can be taken without any danger of changing the composition of the solution due to a continuous loss of camphor from an open tube.

During these operations the bath was brought to a temperature slightly above the melting point of the pure camphor. The end of the sealed tube containing the mixture was immersed in the bath and the contents melted. By quickly removing the tube from the bath, inverting, and giving it a violent shake the contents were completely distributed over the walls of the tube. By again submerging the tube the mixture was melted and run into the bottom of the tube. This process was repeated as often as necessary to insure complete mixing. The tube was then half immersed in the bath. The camphor sublimed onto the upper part of the tube and when the tube was again completely submerged the camphor melted, washing any solute adhering to the side walls into the main body of the solution. By alternate freezing and melting the solution was further mixed.

The actual observations of the freezing and melting points were then taken. The melting point was taken as the temperature at which the last crystal disappeared when the temperature was being raised. The bath temperature was then lowered until the first crystal of camphor reappeared. This temperature was recorded as the freezing point. *Freezing points and melting points were taken alternately until a series of concordant and reliable readings was obtained.* By so doing a good degree of accuracy was obtained. The lag between the two points usually amounted to 1.5–2.0°. The reproducibility of the average of the two temperatures was about 0.1–0.2° however. Since the lowering of the melting point was in the neighborhood of 20° this gave an accuracy of about 1 per cent. The melting point of the camphor was taken in the same manner as that of the solution. Better results were

obtained by taking the average of the melting and freezing points, than by using the temperature at which the last crystal disappeared as in the technique of Rast. It is reasonable that such should be the case too, for the mixing in the melting point tube is not good. This will make the observed melting point too high, because the layer of the solution in the immediate vicinity of the melting camphor will be richer in camphor than the thoroughly mixed solution would be. Carlsohn (7) has also pointed out errors arising when substances are used which are soluble in camphor with difficulty. The freezing point observed, on the other hand, will be too low due to supercooling. The average however will be nearer the true melting point of the camphor in the solution than either of the actual observations. For this reason we have introduced this modification and shown its worth by experimental observations. One precaution needs special emphasis; *viz., the melting point tube during the observations must be completely submerged.*

Calculation.—The molecular weight was then calculated from the accumulated data. The simplest way to explain the use of these data is actually to record one set of data and one calculation.

Freezing Point Constant of Camphor.—The molecular weights were calculated by the usual formula

$$m = K \frac{1000 \, w}{W \, \Delta T_t}$$

where m is the molecular weight of the solute, w is the weight of of the solute, W is the weight of the solvent, ΔT_t is the lowering of the freezing point of camphor observed, and K is the lowering of the freezing point of camphor when 1 mol of solute is dissolved in 1000 gm. of camphor, or the cryoscopic constant for camphor.

It is essential that we have the correct value for K before we can calculate molecular weights by this method. There seems to be some question as to the correct value for this constant, however, since different investigators have published widely differing results. Rast (1) gives the value as 39.7, and Houben (2) uses the closely agreeing value 40.0. M. Jouniaux (8), however, has obtained the average value, 49.5, from a large number of determinations which are not consistent among themselves. Efremov (9) has found 48.5 as the average of his determinations.

Because of the discrepancy in these values it is necessary to evaluate the constant from other data. There are two methods available for the calculation of this constant: first from cryoscopic systems which behave normally throughout, and second from the thermodynamic equation involving the value for the latent heat of fusion. The latter method is perhaps the more accurate and generally useful, because the constant calculated by the use of the latent heat of fusion is not dependent on the specific properties of individual solutions. There are three ways in which the latent heat of fusion may be obtained, exclusive of the freezing point method: first, calorimetrically; second, from vapor pressure data for solid and liquid camphor; and third, from the rate of change of freezing point with pressure. The first of these methods has never been used and the second method is not practicable because of the inaccuracy of the vapor pressure data. This leaves only the third which can be employed. By this method, Hulett (10) found the latent heat of fusion to be 9.4 calories per gm. While this value is not as accurate as we need for this purpose it will serve as a check on the values for K given in the literature.

In searching for cryoscopic systems which behave normally throughout, we found only one system which served our purpose, *viz.* the system camphor-menthol. By using the data for this system given in Landoldt-Börnstein (11) it is possible to verify Hulett's value for the latent heat of fusion from the thermodynamic equation

$$L_f \quad \frac{R \ln \frac{1}{x}}{\left(\dfrac{1}{T_f} - \dfrac{1}{T_{fo}}\right)}$$

assuming L_f, the molal heat of fusion, to be independent of the temperature. x is the mol fraction of solvent in the solution, T_f is the observed freezing point, and T_{fo} is the freezing point of pure camphor. The other symbols have their usual significance. The results of this calculation are shown in Table I. This value is in excellent agreement with Hulett's observation.

If Hulett's value is used and substituted in the thermodynamic equation already given, it will now be possible to calculate the

cryoscopic constant. This can be done by calculating the freezing point lowering for a given mol fraction and then dividing the calculated lowering by the molality. These values are tabulated below.

Molality.	$\frac{1}{x}$	T_f	K
0.1	1.015	4.7	47.0
0.5	1.076	20.0	40.0
1.0	1.153	37.6	37.6

While the numbers in the last column should be corrected for the change of L_f with temperature, this is impossible because the data are not available for the heat capacities of liquid and solid camphor.

From the foregoing calculation it is readily seen that when the lowering is about 20°, the cryoscopic constant is very nearly 40.0,

TABLE I.

x	$\log \frac{1}{x}$	$\frac{1}{T_f}$	$\frac{1}{T_{fo}}$	$\left(\frac{1}{T_f} - \frac{1}{T_{fo}}\right)$	L_f	l_f
0.9264	0.03342	0.002336	0.002231	0.000105	1458	9.58
0.8854	0.05269	0.002407	0.002231	0.000176	1362	9.02
0.7673	0.12156	0.002625	0.002231	0.000394	1414	9.29
0.7035	0.15290	0.002709	0.002231	0.000478	1465	9.63
Average...						9.38

the value given by Rast (1) and Houben (2). For this reason we will continue to use the value published by Rast, $viz.$ 39.7, a value which has been shown to give reliable results by the method in use.

Care must be exercised in applying this constant to all samples of camphor, however, for Datin (12) has shown that camphor from various sources has different vapor pressures. There is great need of a critical investigation of the physical properties of camphor.

Melting Point of Pure Camphor.

In order that the melting point of the camphor would be comparable with that of the solution, the same method of taking and recording the observations was used.

Temperature Readings.		Auxiliary Data.	
Melting point. °C.	Freezing point. °C.		°C.
177.4	176.6	Average temperature of exposed	
177.3	176.5	column.........................	37.5
177.4	176.4	Scale reading at point of immersion.	97.0
177.36	176.50	Stem correction*.................	1.72
Average, 176.93°.		Melting point correction.........178.65	

Melting Point of Solution.

	Material.	
	Thymol. gm.	Camphor. gm.
Weight of tube and substance..............	0.13042	0.16668
" " "	0.12684	0.13042
" " substance......................	0.00358	0.03626

Temperature Readings.		Auxiliary Data.	
Melting point. °C.	Freezing point. °C.		°C.
152.0	148.7	Average temperature of exposed	
152.2	148.7	column......................	43.0
152.0	149.0	Scale reading at point of im-	
152.06	148.80	mersion......................	28.0
Average, 150.43°.		Stem correction*................	2.02
		Melting point correction........152.45	
		Lowering of melting point.......	26.20

$$\text{Molecular weight} = \frac{1000 \times 39.7 \times 0.00358}{0.03626 \times 26.20} = 149.6.$$

Theory = 150.16, per cent error = 0.37.

* Stem correction = $(t - i)(t - a)(0.000154)$, where t is the observed temperature; i is the scale reading at the point of immersion of the thermometer in the bath; a is the average temperature of the exposed column; 0.000154 is the coefficient of expansion of mercury in glass (13).

DISCUSSION.

In Table II, a summary of the results obtained by this method is recorded. The determinations marked with an asterisk were not done in the micro way but followed the original method of Rast; viz., by transferring a small portion of a previously prepared solution to the melting point tube. In some instances it was observed that this procedure had decided disadvantages. The manipulation described here yielded much better results. The work on the molecular weights of carotin and xanthophyll will be described in another place. Duplicate determinations check excellently. There are a few facts which should be noted. From Table II it will be seen that acids show considerable abnormality.

This behavior is being investigated and already interesting results have been obtained. Alkaloids and osazones caused blackening of the melt which showed that reaction had set in. It is interesting that Sadikov and Michailov (4) got reasonably good results with these classes of compounds. Further work needs to be done with these types.

TABLE II.

Results of Determinations on Molecular Weights by the Micro Method.

Substance.	Weight of sample.	Weight of camphor.	Freezing point depression.	Molecular weight observed.	Molecular weight theory.	Error.
						per cent
Naphthalene.*.......	0.0101	0.1390	22.3	129.3	128.1	0.94
α-Naphthol.*........	0.0150	0.2072	19.8	145.0	144.1	0.62
"	0.00291	0.03025	27.01	141.5	144.1	1.80
Hydroquinone.*......	0.0108	0.2008	19.5	109.6	110.1	0.46
Benzoic acid.*.......	0.0114	0.1120	30.2	133.8	122.0	9.7
Succinic "	0.00233	0.03683	15.6	161.0	118.07	35.5
Salicylic "	0.00304	0.02307	36.13	144.80	138.08	4.87
Diphenylamine.......	0.00357	0.02874	29.4	167.8	169.19	0.83
Benzidine...........	0.00347	0.03285	22.35	187.5	184.2	1.8
Thymol..............	0.00358	0.03626	26.20	149.6	150.16	0.37
Acetanilide..........	0.00183	0.02175	24.3	137.4	135.12	1.69
Urethane............	0.00177	0.03102	23.69	95.6	89.06	7.30
"	0.00227	0.03580	26.28	95.8	89.06	7.57
Hexachlorobenzene...	0.00312	0.02695	16.4	280.2	284.73	1.58
p-Nitrotoluene.......	0.00286	0.03069	27.0	137.1	137.1	0.00
Azobenzene..........	0.00255	0.03242	16.87	185.1	182.16	1.65
Dibromobenzene......	0.00402	0.04170	15.9	240.5	235.98	1.92
Brucine..............	0.00580	0.02670	Decomposition.			
Phenylglucosazone....	0.00263	0.02443	"			

* Original method of Rast.

The greatest need in this field of investigation, however, is to find other solvents which have properties similar to those of camphor, so that when abnormal results are obtained in one solvent other solvents may be used. The properties which are essential are large freezing point depression, ease of purification, sharp melting and freezing points, little supercooling, low viscosity in the liquid state, wide solvent powers when melted and none when solid. Other solvents are being investigated in this laboratory

but no substance has been found which is nearly so useful as camphor.

We wish to express our appreciation for the many helpful suggestions made by Dr. H. A. Spoehr.

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THE RELATION BETWEEN OPTICAL ACTIVITY AND THE REDUCING POWER OF GLUCOSE EXCRETED BY RENAL DIABETICS.

By ELIZABETH J. MAGERS and R. B. GIBSON.

(*From the Clinical Chemistry Laboratory of the Department of Internal Medicine, State University of Iowa, Iowa City.*)

Studies of the glucose in blood filtrates and dialysates with reference to the presence of a more reactive form of lower specific rotation have yielded contradictory results in the hands of different investigators (1–16). This may be because of the difficulty in obtaining from blood a glucose solution free from other optically active substances and of sufficient concentration to give reliable results. Assuming that the structure of the glucose molecule is not altered in its passage through the kidney, a study of the sugar excreted by the renal diabetic might give an indication of the type of glucose in the blood without presenting the difficulties encountered in other studies. With this same end in view, Austin and Boyd (17) have compared the reduction and rotation values of sugar excreted by phlorhizinized dogs; they found no indication of any glucose of low optical activity in the urine.

Renal diabetes is understood usually to mean a glycosuria without hyperglycemia or other symptoms of diabetes mellitus (18). Paullin (19) has shown that the carbohydrate metabolism in this condition is normal. He observed the same increase in the respiratory quotient and in the metabolic rate after the ingestion of glucose that Benedict (20) and others have found with normal subjects. On the basis of Lundsgaard's (12) own work, the type of blood glucose in the renal diabetic is the same as in the blood of normal individuals. In the present study the specific rotation and the reducing power of the sugar excreted by two renal diabetics were compared. It was found that the specific rotation of the sugar excreted approximated that of α,β-glucose when the sub-

jects were fasting and after the administration of glucose and of starch.

The patients were brought to the laboratory after a 14 hour fast as for the ordinary sugar tolerance test. An hour's fasting sample of urine was collected, that voided on arrival being discarded. Finger blood was obtained for the determination of the fasting level of the blood sugar during the preliminary hour. After the ingestion of 50 gm. of glucose in 300 cc. of water or of 65 gm. of crackers, the urine was collected hourly and blood samples obtained every 30 minutes for a 3 hour period. Water was given at the beginning of each hour.

The urine was cleared for polarimetric readings by the use of Lloyd's reagent according to Folin and Berglund (21). This method was found to give consistently clearer filtrates for polarimetric work than did either the use of lead acetate or the Folin-Svedberg (22) procedure. A Schmidt and Haensch polarimeter with a three-divided field and reading to 0.01° was used. The cleared urines were read in a 2 dm. tube with light from a Welsbach lamp filtered through 3 per cent dichromate. Readings were made approaching the equalized field from both left and right and each figure given is the average of thirty readings. These were completed within the hour, so that the next urine specimen was taken care of as soon as voided. The reducing substances were estimated on these same filtrates by a modification of the Folin-Berglund procedure described by Gibson, Mitchell, and Larimer (23). The blood sugars were determined by a micro modification of the Folin-Wu method using 0.2 cc. of blood from the finger tip (23).

A brief description of the two patients follows. This is given to establish their condition as that of renal diabetes.

Case L. B., age 32 years, weight 50 kilos, was described by Gibson and Larimer (24) in 1924. She was admitted to the hospital for the first time in 1923 with a glycosuria of 10 years standing. This had been discovered during the first of her two pregnancies. She has never had symptoms of diabetes mellitus excepting the sugar in her urine other than a pruritus. She had dieted only occasionally. A diagnosis of renal diabetes was made in 1923, at which time her fasting blood sugars ranged from 58 to 91 mg. per cent and her blood sugar 2 hours after meals was

always hypoglycemic.   Dietary management failed to control the glycosuria.   The condition is familial, the two cases presented here being sisters and they state that another sister has been known

TABLE I.

Patient L. B.

Glucose, 50 gm. at 0 hour.

Polariscope reading,* *degrees* ..	0.909	1.324	1.647	1.339
Zero point, *degrees*	0.746	0.746	0.746	0.746
Actual rotation, *degrees*	0.163	0.578	0.901	0.593
Specific " "	50.9	53.5	54.2	52.9

* Since the actual readings were made on urine diluted to half its normal concentration, the actual rotation is multiplied by 2 in calculating the specific rotation.

TABLE II.

Patient L. B.

Crackers, 65 gm. at 0 hour.

Hour.................................	0	0.5	1.0	1.5	2.0	2.5	3.0
Blood sugar, *mg. per cent*........	102	172	160	155	125	100	88
Urine volume, *cc*................	352		105		63		24
Urine sugar.							
Reduction value, *per cent*.....	0.24		2.24		3.96		1.23
Rotation " " "	0.23		2.21		3.82		1.16
Urine sugar, *gm*.................	0.84		2.35		2.49		0.29
Polariscope reading, *degrees*.....	0.816		1.859		2.705		1.309
Zero point, *degrees*..............	0.697		0.697		0.697		0.697
Actual rotation, *degrees*	0.119		1.162		2.008		0.612
Specific " "	49.1		51.8		50.6		49.6

to have glycosuria for 12 years.   The small daughter of L. B. and a son of H. B. have both shown traces of sugar in the urine when tested in this laboratory.

Case H. B., age 29 years, weight 46 kilos, is a sister of Mrs. L. B. She has known of her glycosuria since her first pregnancy 9 years ago. A diet was prescribed for her at that time, but as the glycosuria did not clear up and as she felt as well or better without

TABLE III.

Patient H. B.

Glucose, 50 gm. at 0 hour.

Polariscope reading, *degrees*	1.258	1.691	1.985	1.335
Zero point, *degrees*	0.710	0.710	0.710	0.710
Actual rotation, *degrees*	0.548	0.981	1.275	0.625
Specific " "	49.8	51.6	50.2	49.6

TABLE IV.

Patient H. B.

Crackers, 65 gm. at 0 hour.

Hour....................................	0	0.5	1.0	1.5	2.0	2.5	3.0
Blood sugar, *mg. per cent*	67	123	87	110	79	102	66
Urine volume, *cc*................	115		55		75		75
Urine sugar.							
Reduction value, *per cent*	0.58		2.68		1.88		1.18
Rotation " " "	0.55		2.69		1.86		1.16
Urine sugar, *gm*.................	0.66		1.47		1.41		0.88
Polariscope reading, *degrees*	1.000		2.126		1.686		1.322
Zero point, *degrees*..............	0.710		0.710		0.710		0.710
Actual rotation, *degrees*	0.290		1.416		0.976		0.612
Specific " "	50.0		52.9		51.9		51.9

diabetic management, she discontinued it. She has had no symptoms of diabetes mellitus other than glycosuria.

The blood sugar curves of L. B. (Tables I and II) show a return to the fasting level in 2.5 hours and are to be regarded as not

indicating an essentially altered carbohydrate metabolism (25). As compared with earlier curves obtained on this patient (24), the fasting blood sugar figures are somewhat higher and the descent from the peak is less abrupt. Sugar tolerance curves on H. B. have not been made previously. Both the curve with glucose, and that when crackers were given, are unusual (Tables III and IV). A delayed peak with a rapid fall to a hypoglycemic level occurred with the glucose, while a low peak and a pendulum effect followed the ingestion of crackers. We were unable to persuade this patient to return for further study.

It will be noted that the excretion of sugar by these two patients for the hour preceding the ingestion of carbohydrate is considerable, 0.66 to 1.11 gm. The specific rotation of the fasting glucose varies between 49.1–50.9° with an average for the four periods of 49.9°.

The urinary sugar is increased two to four times after the administration of glucose and crackers. Figures for the specific rotation range from 50.2–54.2° with an average of 52.0° following glucose ingestion and 51.5° after crackers were eaten.

It would seem reasonable to expect that the specific rotation of the sugar of the fasting urine would represent more exactly the normal sugar of the blood than that obtained after the ingestion of carbohydrates. A tendency only to slightly lower figures for the fasting samples was found, and might be expected in view of the presence in small amounts of both levo-rotatory substances and optically inactive reducing bodies which would be in a greater ratio to the per cent of sugar than in subsequent urine specimens. The specific rotation of the sugar excreted both during the fasting state and after the ingestion of glucose and of crackers so closely approaches 52.5° that it may be regarded as α,β-glucose.

SUMMARY AND CONCLUSIONS.

1. The glucose excreted by two renal diabetics was studied with the idea that it would give an indication of the type of glucose normally present in the blood.

2. The glucose excreted in the fasting condition had essentially the same specific rotation as that eliminated during hyperglycemia produced by carbohydrate ingestion.

3. The specific rotation of the sugar excreted by the renal diabetics approximated that of α, β-glucose at all times.

4. The results do not support the idea of the existence in blood of a glucose of low specific rotation.

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BLOOD AS A PHYSICOCHEMICAL SYSTEM.

VI. THE COMPOSITION AND RESPIRATORY EXCHANGES OF HUMAN BLOOD IN TERMINAL CHRONIC NEPHRITIS.

By L. J. HENDERSON, A. V. BOCK, D. B. DILL,* L. M. HURXTHAL, and C. van CAULAERT.

(*From the Medical Laboratories of the Massachusetts General Hospital, Boston.*)

The composition and respiratory function of human blood undergo no more profound changes than in terminal chronic nephritis. Coincidently in this condition red cells and hemoglobin may be reduced to a third or a fourth, bicarbonate to a fifth of the normal amount, and chloride concentration may fall 10 or even 20 per cent. Meanwhile the end-products of nitrogen metabolism may be increased ten- or even twentyfold and the concentration of acids other than carbonic, hydrochloric, and the protein radicals may rise in an important degree. These facts are known chiefly as the result of the investigations of Bulger, Peters, Eisenman, and Lee (1). Such changes in the physicochemical system are necessarily accompanied by changes in its physiological activity.

The present investigation consists of a study of the important physiological variables in the blood of terminal chronic nephritis and of a nomographic representation of the results. A comparison is also made with the blood of normal man at rest, described most recently in Paper V of this series (2).

The observations have been more limited than in the case of normal man. In all, four samples of blood were obtained, two from each of two cases. Venous blood was drawn from A. T. about 18 hours before death and blood was drawn from the heart of the same subject 15 minutes after death. Simultaneous samples of venous and of arterial blood were drawn from H. T. 24 hours before death.

* National Research Fellow in Chemistry.

TABLE I

Oxygen Dissociation Curves in Terminal Chronic Nephritis.

Blood drawn from the heart of A.T. 15 minutes after death.   Oxygen capacity = 5.60 volumes per cent.

pCO_2	pO_2	Total O_2 content.	HbO_2 content.	Oxygen saturation.
mm. Hg	*mm. Hg*	*vol. per cent*	*vol. per cent*	*per cent*
1.1	33.6	3.95	3.87	69.2
40.2	35.1	2.07	1.99	35.6
40.6	46.0	2.89	2.78	49.7
40.2	61.2	3.73	3.58	64.0
78.6	76.6	2.91	2.71	48.5

TABLE II.

Carbon Dioxide Dissociation Curves in Terminal Chronic Nephritis.

Subject.	Remarks.	Oxygen capacity.	pCO_2	pO_2	Total CO_2 content.	
					Whole blood.	True plasma.
		vol. per cent	*mm. Hg*	*mm. Hg*	*vol. per cent*	*vol. per cent*
A. T.	18 hrs. before death; venous blood.	7.18	19.7	Air.	12.43	
			43.0	"	19.3	
			67.2	"	23.7	24.6
			21.2	2.6	13.0	
			41.4	2.9	20.5	19.5
			67.9	2.4	24.7	
	15 min. after death; blood drawn from heart.	5.60	14.7	Air.	8.4	8.6
			92.8	"	24.3	23.7
			28.0	2.8	14.9	14.9
			100.2	2.9	25.9	25.6
H. T.	24 hrs. before death; arterial blood; CO_2 content, 18.3 vol. per cent.	8.44	19.4	Air.	15.0	
			51.8	"	25.8	
	Simultaneous sample of venous blood.	8.56	40.5	Air.	22.2	23.2
			47.5	6.0	25.5	26.7

The sample of cardiac blood had a cell volume less by one-half than that of the venous blood taken 18 hours earlier from the same patient, a modification which we attribute to changes in the

circulation. The composition of both plasma and cells of the two samples was similar, suggesting that as the circulation slowed down the corpuscles were skimmed off in the small vessels. Accordingly this cardiac sample was concentrated by removing part of the serum after centrifugation. The resulting blood, with a cell volume of 14.7 per cent, furnishes a picture of the respiratory

TABLE III.

Miscellaneous Observations on Blood in Terminal Chronic Nephritis.

	A.T. 18 hrs. before death; venous blood.	A.T. 15 min. after death; cardiac blood.	H.T. 24 hrs. before death; venous blood.
H_2O *cc. per l. serum*	952.0		947 0
H_2O " " " *cells*	752.0		728 0
Cl *mM* " " *blood*			94.9
" " " " *serum*	91.8	93.0	103.5
" " " " *cells*	65.5		
" " " " *spinal fluid*		121.5	
Arterial pCO_2, *mm. Hg*			27.4*
Oxygen capacity, *vol. per cent*	7.18	5.60	8 56
Cell volume, *per cent*	18.7		21.0
Total nitrogen, *gm. per l. serum* ...	13.17	12.15	12 21
Non-protein " " " " "	4.70	3.80	3 65
Protein " " " " "	8.47	8.35	8 56
" *gm. per l. serum*	53.0	52.2	53.5
Uric acid, *mg.* " " "		136 0	
Creatinine " " " "		99.0	
Phosphorus " " " "		238.0	
Base, *mM* " " "		157.1	
" " " " *spinal fluid.*		148.0	

* This observation is based upon arterial blood.

medium of a nephritic as death intervenes. Part of this concentrated sample was analyzed to provide data for the oxygen dissociation curves. The results are shown in Table I.

Portions of each of the four specimens were also equilibrated with suitable pressures of oxygen and of carbon dioxide in order to obtain information concerning the carbonic acid capacity. The equilibrated whole blood and true plasma were analyzed in the

TABLE IV.

Blood of a Nephritic at Death.

Concentration of hemoglobin = 2.50 mM per liter of blood.
    " serum proteins = 44.6 gm. " " "
    Respiratory quotient = .73

	Arterial.			Venous.			Δ		
	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.	Serum.	Cells.	Whole blood.
H_2O cc. per l. blood	812.0	110.5	922.5	810.9	111.6	922.5	−1.1	+1.1	0.0
B mM " " "	134.0	15.0	149.0	134.0	15.0	149.0	0.0	0.0	0.0
X " " " "	46.35	1.55	47.90	45.80	2.18	47.98	−0.55	+0.63	+0.08
Cl " " " "	78.37	9.63	88.00	78.30	9.70	88.00	−0.07	+0.07	0.0
BP " " " "	6.40	3.30	9.70	6.33	2.49	8.82	−0.07	−0.81	−0.88
$BHCO_3$ " " " "	2.88	0.52	3.40	3.57	0.63	4.20	+0.69	+0.11	+0.80
" vol. per cent	6.44	1.17	7.61	8.00	1.41	9.41	+1.56	+0.24	+1.80
H_2CO_3, mM per l. blood	0.39	0.05	0.45	0.52	0.07	0.59	+0.13	+0.02	+0.15
" vol. per cent	0.88	0.12	1.00	1.16	0.16	1.32	+0.28	+0.04	+0.32
Total CO_2, mM per l. blood	3.27	0.58	3.85	4.09	0.70	4.79	+0.82	+0.12	+0.94
" vol. per cent	7.32	1.29	8.61	9.16	1.57	10.73	+1.84	+0.28	+2.12
Free O_2, mM per l. blood			0.12			0.04			−0.08
" vol. per cent			0.28			0.09			−0.19
Combined O_2, mM per l. blood		2.37	2.37		1.16	1.16		−1.21	−1.21
" vol. per cent		5.32	5.32		2.60	2.60		−2.72	−2.72
Total O_2, mM per l. blood			2.49			1.20			−1.29
" vol. per cent			5.60			2.69			−2.91
pCO_2, mm. Hg			15.0			19.7			+4.7
pO_2, " "			110.0			35.0			−75.0
Volume, cc. per l. blood	853.0	147.0	1000.0	851.9	148.1	1000.0	−1.1	+1.1	0.0
pH	6.994	6.987		6.969	6.970		−0.025	−0.017	
r_{HCO_3}			1.33			1.28			−0.05
r_{Cl}			0.903			0.900			−0.003

usual manner and from the results, shown in Table II, carbon dioxide dissociation curves were constructed.

Miscellaneous observations on the two samples of the blood of A. T. and on the venous blood of H. T. are collected in Table III.

With these data the nomogram, Fig. 1, was constructed in the usual manner.   As far as possible this represents the blood of A. T.

TABLE V.

Serum of a Nephritic at Death.

					Arterial.	Venous.	Δ
H_2O	cc.	per l. serum		....	952.2	952.0	−0.2
B	mM	" "	"	....	157.1	157.4	+0.3
X	"	" "	"	....	54.4	53.8	−0.6
Cl	"	" "	"	....	91.8	92.0	+0.2
BP	"	" "	"	....	7.5	7.4	−0.1
$BHCO_3$	"	" "	"	....	3.4	4.2	+0.8
H_2CO_3	"	" "	"	....	0.5	0.6	+0.1
Total CO_2	"	" "	"	....	3.9	4.8	+0.9

TABLE VI.

Cells of a Nephritic at Death.

					Arterial.	Venous.	Δ
H_2O	cc.	per l. cells		....	751.7	753.3	+1.6
B	mM	" "	"	....	102.0	101.3	−0.7
X	"	" "	"	....	10.5	14.7	+4.2
Cl	"	" "	"	....	65.5	65.5	0.0
BP	"	" "	"	....	22.5	16.8	−5.7
$BHCO_3$	"	" "	"	....	3.5	4.2	+0.7
H_2CO_3	"	" "	"	....	0.3	0.5	+0.2
Total CO_2	"	" "	"	....	3.8	4.7	+0.9
Combined O_2	"	" "	"	....	16.1	7.8	−8.3
Total Hb	"	" "	"	....	17.01	16.89	−0.12

at the time of death.   In some particulars, where direct observations are lacking, we have used the data of premortem samples to guide us in making approximations.   Thus the r for chloride and for bicarbonate is a composite figure.   The r for hydrogen ion is merely a guess based in part on the theoretical calculations of Van Slyke, Wu, and McLean (3) and in part on the experimental observations of Van Slyke, Hastings, Murray, and Sendroy (4).

Table IV presents the facts which we have been able to deduce concerning the composition of arterial and venous blood and the respiratory exchanges in A. T. at the time of death.   Tables V and VI give the composition of serum and cells under the same

TABLE VII.

Arterial Serum of A. V. B. at Rest and of a Nephritic at Death.

		A.V.B.	Nephritic.	Δ
H₂O	*cc. per l. serum*...............	943.3	952.2	+8.9
B	*mM* " " " "	154.00	157.10	+3.10
X	" " " " "	17.00	54.40	+37.40
Cl	" " " "	99.32	91.80	−7.52
BP	" " " "	13.13	7.50	−5.63
BHCO₃	" " " "	24.55	3.40	−21.15
H₂CO₃	" " " "	1.22	0.50	−0.72
Total CO₂	" " " "	25.77	3.90	−21.87
pH....................................		7.425	6.994	−0.431
Protein, *gm. per l. serum*................		72.5	54.9	−17.6

TABLE VIII.

Arterial Cells of A. V. B. at Rest and of a Nephritic at Death.

		A.V.B.	Nephritic.	Δ
H₂O	*cc. per l. cells*.............	705.0	751.7	+46.7
B	*mM* " " "	133.75	102.00	−31.75
X	" " " "	17.73	10.50	−7.23
Cl	" " " "	45.27	65.50	+20.23
BP	" " " "	56.50	22.50	−34.00
BHCO₃	" " " "	14.25	3.50	−10.75
H₂CO₃	" " " "	0.93	0.30	−0.63
Total CO₂	" " " "	15.18	3.80	−11.38
Combined O₂	" " " "	21.43	16.10	−5.33
Total Hb	" " " "	22.33	17.01	−5.32
pH	....	7.124	6.987	−0.137

conditions.   Tables VII and VIII present a comparison of the serum and cells of this case with the normal.

In most respects the profound pathological changes are plainly evident even from a casual inspection of the above nomogram and tables.   The volume of cells is little more than one-third normal,

the amount of hemoglobin even lower, or hardly three-tenths normal. This corresponds to a concentration of hemoglobin in the cells of only about three-fourths the normal value. The hydrogen ion concentration of cells and plasma in both arterial and venous blood is greater than $1 \times 10^{-7} N$. Accompanying these changes there is an unprecedented change in the distribution of electrolytes between the two phases. Venous cells are no more acid than plasma; the concentration of bicarbonate ion in cells is much greater, the concentration of chloride ion hardly less, than the concentration in plasma. Total carbonic acid concentration is but one-fifth of the normal, total base bound by protein hardly more. Thus all the elements of the acid-base equilibrium, including the mechanism of the transport of carbonic acid, the mechanism of oxygen transport, and the heterogeneous equilibrium of cells and plasma, are greatly deranged.

Under these conditions, even with lowered metabolism, blood flow and coefficient of utilization must be increased, and no factor of safety remains. Thus a considerable increase in the metabolism is impossible. We have, in fact, a complete picture of a physiological mechanism at the point of dissolution. Yet adaptive properties of these changes are not entirely lacking. For instance, the high value of the hydrogen ion concentration tends in part to reduce anoxemia and the diminished serum chloride is probably the result of a process by which the acidosis has been held in check and serves at least slightly to diminish the osmotic pressure. But on the whole there can be little doubt that these properties of blood only barely afford the necessary conditions for the existence of the individual.

<div align="center">SUMMARY.</div>

This paper consists of a nomographic description of the blood and respiratory changes in chronic nephritis at the point of death.

<div align="center">*Case Histories.*</div>

A. T., No. 230752.—A man 32 years old had complained of headaches occurring once or twice daily for as long as he could remember. The past history was otherwise negative. 4 weeks before admission he had a sore throat for 3 days. Purple spots about 2 cm. in diameter appeared on his legs and lasted about 1 week. Epistaxis and vomiting occurred almost every day for 2 weeks. He had had no convulsions and no edema. He had

been drowsy, the usual headaches continued, there was blurred vision and weakness. His face was moderately swollen. There were purpuric spots on both legs and the mucosœ were pale. The nose was obstructed, tonsillitis was present, and the breath was foul. The lungs were clear. There was slight cardiac enlargement and a systolic murmur at the apex. The blood pressure was 160/90. The urine contained a large trace of albumin and many red blood cells. The non-protein nitrogen on the 2nd day was 470 mg. per 100 cc. of blood. The patient died 4 days after admission. Necropsy showed chronic glomerulonephritis; hypertrophy and dilatation of the heart; septicemia (*Streptococcus hæmolyticus*); intussuception (ileum into cecum) and diphtheritic colitis.

H. T., No. 231258.—A man 55 years old had had acute nephritis 20 years before admission. For the year before admission he noticed weakness which increased a month before admission, and was accompanied by shortness of breath. His appetite had been failing and his sleep poor. He gave up his work 2 days before entry and had vomited after every meal since then. He was poorly developed and nourished, sallow, breathed stertorously, and was semicomatose. His breath had a urinous odor. His pupils were small and reacted poorly. His heart was markedly enlarged with a systolic murmur over the aortic area and apex. The blood pressure was 150/80. The abdomen was negative. There was marked twitching of the facial muscles. There was slight edema of the feet. The urine contained a large trace of albumin and the non-protein nitrogen was 300 mg. per 100 cc. of blood.

The final diagnosis was chronic nephritis with hypertension; hypertensive heart disease; secondary anemia. The patient died 24 hours after entry.

These two cases apparently developed nephritis from different etiological causes, but at the time of death presented indistinguishable clinical appearances.

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LACTONE FORMATION OF GALACTOARABONIC AND OF MELIBIONIC ACIDS AND ITS BEARING ON THE STRUCTURES OF LACTOSE AND OF MELIBIOSE.

BY P. A. LEVENE AND OSKAR WINTERSTEINER.[*]

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

Levene and Sobotka[1] have pointed out that from the progress of lactone formation of a given bionic acid conclusions may be drawn regarding the place of union of the two components. Thus, if position (4) is the place of union, then only a $<1, 5>$ lactone can be formed; if position (5) is substituted, only a $<1, 4>$ lactone can be formed. If the substitution is neither in (4) nor in (5), then the bionic acid will form two lactones. From the curve of mutarotation of the acid it is possible to judge as to the course of lactone formation. Thus, theoretically, it seems possible to obtain much information on the structure of a disaccharide from the observations of the lactone formation in the corresponding bionic acid.

Levene and Sobotka have shown that the observations on the lactone formation of lactobionic acids led to the same conclusion regarding the structure of lactose as the more complicated method of Zemplén.[2] In order to test the method more rigorously it seemed desirable to apply it to a disaccharide of known structure in which the positions (4) and (5) of the substituted constituent were free. A disaccharide of this structure was prepared by degradation of lactose into galactoarabinose. Inasmuch as in lactose the glucose is substituted in position (4), then in galactoarabinose the arabi-

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[*] Fellow of the International Education Board.

[1] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1926–27, lxxi, 471.

[2] Zemplén, G., *Ber. chem. Ges.*, 1926, lix B, 2402.

nose is substituted in position (3).   Hence, it should be expected that galactoarabonic acid would show a mutarotation curve resembling that of unsubstituted sugar acids.   The results of the observations on the lactone formation of galactoarabonic acid fully agreed with the expectation.   In the early phases of the experiment, the dextrorotation of the solution dropped markedly and then slowly and gradually increased.   The second phase of the mutarotation curve was due to $<1,4>$ lactone formation and not to hydrolysis.   The absence of the latter was demonstrated by the negative reduction test.   On the other hand, the titration value showed a gradual drop.

Thus, comparing the results of observations on lactone formation in lactobionic and in galactoarabonic acids, it is evident that the lactone formation in bionic acids proceeds similarly to that in sugar acids substituted by simple groups such as methyl, and hence that in sugar acids of unknown structure the course of lactone formation may serve as a basis for revealing the place of substitution.

The method was then applied to melibiose.   In regard to melibiose two theories have been advanced, one by Haworth and Leitch,[3] attributing to the disaccharide the following structure

CHOH———┐   ┌———CH———┐
| | | |
HCOH | | HCOH |
| | | |
HOCH   O   O   HOCH   O
| | | |
HCOH | | HOCH |
| | | |
HC————┘   └   HC————┘
| |
CH₂————————┘   CH₂OH
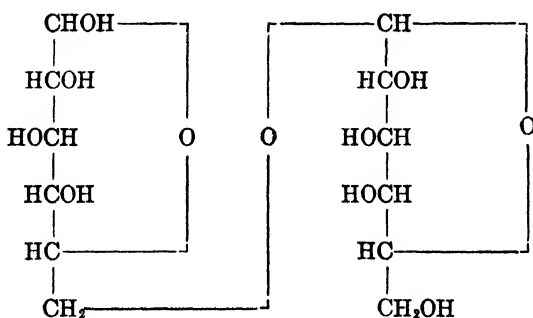
and the other by Zemplén,[4] assuming the substitution in position (3) of the glucose as follows:

[3] Haworth, W. N., and Leitch, G. C., *J. Chem. Soc.*, 1918, cxiii, 189.

[4] Zemplén, G., *Ber. chem. Ges.*, 1927, lx B, 923.

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CHOH──────┐      O─────────CH-
   │           │               │
 HCOH          │             HCOH
   │           │
  CH          O            HO           O

 HCOH                      HOCH ·

 HC─────────────┘          HC-

 CH₂OH                     CH₂OH
```
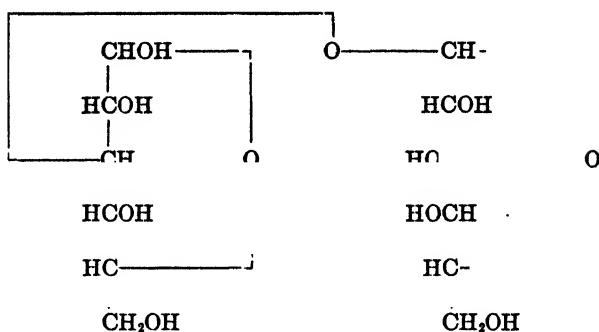
According to either one of the two theories, the lactone formation of melibionic acid should follow the same course as in the case of galactoarabonic acid and not as in the case of lactobionic acid. In our experiments, however, the course of lactone formation of melibionic acid was the same as in the case of the latter, and on this basis, the conclusion seems to follow that in melibiose the substitution takes place in position (4) of the glucose. We realize that because of the disagreement reached by the different methods of work, the investigation of the English authors as well as that of Zemplén should be repeated.

<div align="center">

EXPERIMENTAL.

</div>

Preparation of Calcium Galactoarabonate from Galactoarabinose.

Galactoarabinose was prepared from lactose according to Zemplén by way of lactose oxime and octacetyllactobionic nitrile, and the sugar obtained after splitting off the cyanide group by means of sodium methylate was isolated in the form of the benzylphenylhydrazone (m.p. = 220–221°, after recrystallization from 80 per cent alcohol, Zemplén 222°).

The substance analyzed as follows (micro Dumas).

5.610 mg. substance: 0.285 cc. N₂ (761 mm., 26°C.).
$C_{24}H_{23}N_2O_9$. Calculated. N 5.69.
                 Found.   " 5.73.

The hydrazone was converted into the free sugar by means of benzaldehyde. The resulting solution containing 10.9 gm. of sugar and a small amount of phenylhydrazine, not removable

with ether, was submitted to oxidation to the corresponding bionic acid by the method of Goebel.[5]

A small fraction of the solution was evaporated under reduced pressure to a thick syrup, freed from water by continuous evaporation with alcohol, and dried to constant weight over P_2O_5.

$$[\alpha]_D^{28} = \frac{-2.47° \times 5.0699}{2.339 \times 1.0117} = -52.9°.$$

(Zemplén $[\alpha]_D = -55°, -58°$.)

The reduction (Willstätter and Schudel)[6] was found higher than the result of the direct determination by weight indicated, due to the presence of small amounts of phenylhydrazine.

320 cc. of a solution containing 10.9 gm. of galactoarabinose were oxidized with barium hypoiodite in two equal portions. Thus each contained 5.45 gm. (0.0176 mol). A solution of 10.35 gm. of iodine and 20.5 gm. of barium iodide was prepared, combined with the sugar solution (5.45 gm. sugar), and a solution of 25 gm. of barium hydroxide (ten times recrystallized) in 400 cc. of water was added over an interval of 5 minutes under mechanical stirring. The colorless liquid was allowed to stand 15 minutes, then under rapid stirring 7.5 cc. of concentrated sulfuric acid dissolved in 100 cc. of water were added and immediately 90 gm. of lead carbonate stirred in to neutralize the hydriodic acid. The reaction toward Congo red after about 1 minute's stirring was neutral, thus showing that the reaction was completed. After the precipitate of barium sulfate, lead sulfate, and lead iodide had settled, the solution was filtered and the precipitate washed several times with water, the washings were combined with the filtrate, and evaporated under reduced pressure to about 300 cc. Small amounts of lead iodide were filtered off, and to the filtrate, which was nearly colorless and contained the acid in the form of the lead salt, the calculated amount of sulfuric acid plus a small excess was added in order to remove the lead. Traces of hydroiodic acid still present in the liquid were removed by means of silver sulfate. The silver ions were removed from the filtrate by means of hydrogen sulfide. In the final filtrate the sulfuric acid was removed

[5] Goebel, W. F., *J. Biol. Chem.*, 1927, lxxii, 801.

[6] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, 1918, li, 780.

quantitatively by means of barium hydroxide, and the solution, which was then free from all inorganic constituents, boiled with an excess of calcium carbonate. If necessary a small amount of norit may be added to clear the solution. After filtering, the colorless liquid was evaporated under reduced pressure to a syrup and poured into 12 to 15 volumes of absolute methyl alcohol.

The calcium salt of the galactoarabonic acid was precipitated as a white, partly crystalline mass, whereas contaminating reducing substances, which probably had formed in small amounts during the oxidation with barium hypoiodite, were to a large extent held in solution. For purification the crude salt was dissolved in about 50 cc. of water and about $1\frac{1}{2}$ volumes of methyl alcohol were added. The solution turned very slightly opalescent and after a short time the salt began to crystallize. The crystallization was completed in a few hours. This operation was repeated once. The crystalline salt still contained water of crystallization which was partly given off at 125°C. under reduced pressure after a long period of drying. The analytical results, however, obtained on this product dried at high temperature indicated that it still contained 1 molecule of water which could not be removed without decomposition of the substance. Yield of pure salt was about 45 per cent of the theory.

5.121 mg. substance	0.961 mg. $CaSO_4$,	5.52 per cent Ca.
7.023	1.384	5.80
7.450	1.413	5.58
8.580	1.674	5.74
7.932	1.444	5.69
12.114	2.330	5.66
13.800	2.634	5.62

Calculated for $(C_{11}H_{19}O_{11})_2 \cdot Ca + 1\ H_2O$. Ca 5.63 per cent.

5.402 mg. substance: 2.84 mg. H_2O, 7.38 mg. CO_2, 5.88% H, 37.26% C.
4.475 "   "   : 2.28 "   "   6.08 "   "   5.57% " 37.05% "

Calculated for $(C_{11}H_{19}O_{11})_2 \cdot Ca + 1\ H_2O$. H 5.66, C 37.06.

The rotation of the calcium salt was as follows:

$$[\alpha]_D = \frac{+\ 0.62° \times 100}{1 \times 1.84} = +33.6°.$$

Mutarotation Experiment.—0.9669 gm. of calcium galactoarabonate (dry substance, corresponding to the salt + 1 mol of

H_2O) were dissolved in a few cc. of water, the calculated amount of 0.5 N hydrochloric acid added (accurate to 0.01 cc.), and the volume quickly made up to 25 cc. The change of the rotation was followed in a 2 dm. tube and at the same time, samples of 1 cc. were titrated with 0.1 N sodium hydroxide (phenolphthalein). The samples were taken with a very accurate pipette, holding

TABLE I.*

Changes in Rotation of Galactoarabonic Acid Solution.

$l = 2$ dm. $t = 34°$ λ $= 5892$ Å.

Experiment 1.		Experiment 2.	
Time.	α	Time.	α
min.	*degrees*	*min.*	*degrees*
0	+2.27	0	+2.39
15	+2.12	16	+2.19
30	+2.08		
45	+2.08	41	+2.10
hrs.		*hrs.*	
1	+2.07		
1¼	+2.08	1⅓	+2.10
1½	+2.11		
2	+2.13	2	+2.15
2½	+2.16		
3	+2.19	3	+2.20
4	+2.28		
5	+2.33	5	+2.30
6	+2.38		
7	+2.44	8	+2.39
11	+2.60	11½	+2.50
21	+2.80		
24	+2.91	24	+2.85
28	+2.98		

* Each series of rotations was made by a different observer.

exactly 1.0005 cc., which was washed out twice with carbon dioxide-free water. The data are given in Tables I and II.

The solution contained 0.0356 gm. of free galactoarabonic acid, the molecular weight of which is 328. The equivalent weight as indicated by the first titration value was found to be 349 in Experiment 1, and 337 in Experiment 2.

5 cc. of the solution used for the rotation experiment were titrated according to the Willstätter-Schudel method after the last reading (28 hours), but no reduction was found either in this sample or in the unconverted calcium salt of which a corresponding amount was titrated in the same way, proving that no hydrolysis of the bionic acid had taken place during the experiment.

The specific rotation of the free acid, based upon the first reading in the rotation experiment, was

$$[\alpha]_D^{24} = \frac{+\ 2.27° \times 100}{2 \times 3.56} = +\ 31.9°.$$

TABLE II.*

Titration of 1,000 Cc. of Galactoarabonic Acid Solution with 0.1 N Sodium Hydroxide.

Experiment 1.		Experiment 2.	
Time.	0.1 N NaOH.	Time.	0.1 N NaOH.
hrs.	cc.	hrs.	cc.
½	1.02	0	1.06
1	1.02		
1½	1.02	2	1.00
2½	0.98	4	0.95
5½	0.95	5½	0.92
8	0.92		
28	0.51	20½	0.78

* Each series of titrations was made by a different observer.

Melibionic Acid.

30 gm. of crude raffinose were fermentated in about 10 per cent solution with 1 gm. of bakers' yeast and a small amount of malt sprouts at room temperature. After 48 hours the rotation became constant. The solution was treated with basic lead acetate, the lead removed from the filtrate by means of hydrogen sulfide, and, after clearing with norit, the solution was concentrated under reduced pressure to a syrup. The specific rotation and the reducing power were determined on samples dried to constant weight over phosphorus pentoxide.

$$[\alpha]_D^{20} = \frac{+\ 5.79° \times 5.07}{1 \times 0.2465 \times 1.014} = +\ 117.4°.$$

0.1885 gm. required 9.8 cc. 0.1 N iodine solution (Willstätter-Schudel) = 0.168 gm. melibiose.

Total amount of melibiose as calculated from reducing power: 8.25 gm. of melibiose (anhydrous) = 0.0241 mol.

To the syrup (21 cc.) a solution of 12.2 gm. of iodide (0.0964 equivalent) and 25 gm. of barium iodide in 300 cc. of water were added. (The concentration of iodine is about 0.3 N.) A solution of 22.8 gm. of crystalline barium hydroxide (0.0723 mol) in 360 cc. of water (about 0.4 N) was added to the above solution over an interval of 5 minutes in a constant flow, under mechanical stirring. The liquid was allowed to stand for 15 minutes; then under rapid stirring 8.15 cc. of concentrated sulfuric acid dissolved in about 60 cc. of water were added and 100 gm. of lead carbonate were immediately poured in. After about 2 minutes the solution became neutral to Congo red. The precipitates of barium sulfate and lead iodide were allowed to settle, and the supernatant liquid was poured off and combined with the washings of the precipitate obtained by repeated centrifugalization with water. The liquid was freed from iodine by distillation under reduced pressure and evaporated to about 200 cc. It was filtered, and a small excess of sulfuric acid sufficient to precipitate the lead ions was added. Small amounts of hydriodic acid were then removed by addition of silver sulfate. The silver ions were removed by means of hydrogen sulfide. The filtrate was freed from sulfate ions by means of barium hydroxide and then shaken with an excess of calcium carbonate for 15 minutes. Short boiling completed the formation of the calcium salt. The solution was filtered after cooling and evaporated to a syrup, which was poured into 10 to 15 volumes of absolute methyl alcohol. The semicrystalline crude product was purified by dissolving in about 50 cc. of water and adding an equal amount of methyl alcohol, whereupon it settled out in fine large crystals. By repeating this procedure, a pure product was obtained which was dried over calcium chloride. The salt thus obtained contained 11.25 per cent of water which could be driven off by prolonged drying at higher temperature. The salt then analyzed as follows:

8.943 mg. substance: 1.627 mg. $CaSO_4$, 5.36 per cent Ca.
7.370 "   "   : 1.361 "   "   5.43 "   "   "
Calculated for $(C_{12}H_{21}O_{12})_2$. Ca 5.31 per cent.

0.0942 gm. substance: 0.0494 gm. H_2O and 0.1318 gm. CO_2.
Calculated. H 5.61, C 38.18.
Found.   " 5.86, " 38.15.

Mutarotation and Titration Experiments.

1.0788 gm. of calcium melibionate containing 11.25 per cent of water, thus corresponding to 0.9575 gm. of dry substance, were dissolved in about 10 cc. of water. The calculated amount of 0.5 N

TABLE III.*

Changes in Rotation of Melibionic Acid Solution.

$l = 2$ dm. $t = 32°$ $\lambda = 5892$ Å.

Experiment 1.		Experiment 2.	
Time.	α	Time.	α
min.	*degrees*	*min.*	*degrees*
0	+7.92	0	+7.50
5	+7.97		
15	+8.01		
30	+8.01	25	+7.57
45	+8.02	41	+7.60
hrs.		*hrs.*	
1	+8.04		
1½	+8 05	1½	+7.61
2	+8.03		
3	+8.06	3	+7.63
4	+8.06	4½	+7.63
6½	+8.12	6½	+7.63
10	+8.15	10·	+7.65
13	+8.17	19	+7.70

* Each series of rotations was made by a different observer.

hydrochloric acid was added and the solution was made up to 25 cc. The change in rotation was observed and samples of 1.0 cc. titrated at suitable intervals with 0.1 N sodium hydroxide (phenolphthalein). The amount of sodium hydroxide required for neutralization remained constant through the whole experiment, namely 1.00 cc. Since 0.0364 gm. of melibionic acid was present in 1 cc., this value is in close agreement with the theory, which demands 0.0358 gm. in 1 cc. of a 0.1 N solution of the acid.

In the second experiment 1.0 gm. of the calcium salt containing 0.862 gm. of the anhydrous salt was dissolved in 25 cc. of water and an equivalent of hydrochloric acid was added. The changes in rotation were followed as in the first experiment. On titration 1 cc. was equivalent to 0.9 cc. of 0.1 N alkali, which corresponds to the molecular weight of 363 against 358 required by the theory. The titration value remained constant. It was measured at 2 hour intervals for 12 hours and the last titration was made after 24 hours. The data are given in Table III.

The specific rotation based upon the first readings of the rotation experiment is

$$[\alpha]_D^{n} = \frac{+\ 7.92° \times 100}{2 \times 3.64} = +\ 108.8°.$$

ON CONDENSATION PRODUCTS OF PROPYLENE OXIDE AND OF GLYCIDOL.

BY P. A. LEVENE AND A. WALTI.

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York.*)

One of the problems of organic chemistry which engages the attention of the workers of today is that of the structure of naturally occurring substances of high molecular weight such as polysaccharides, proteins, etc. Whereas classical organic chemistry viewed the formation of the complex from the simple molecules as a process of condensation through the well recognized forces of primary valence, present day chemists are inclined to the view that the simpler components are linked to each other by the forces of residual valence due to incomplete saturation of groups or elements within the simpler molecules. In the polysaccharides, such residual affinities are attributed principally to the oxidic ring. Considerable evidence has been advanced in favor of this view by Bergmann,[1] Karrer,[2] Pictet,[3] Pringsheim,[4] and others. Still the problem as yet cannot be regarded as definitely settled.

From this view-point it seems important to investigate the simple cyclic compounds with respect to their tendency to polymerize or to condense with one another. Taking for example ethylene oxide, the simplest substance of this group, it is evident that it may polymerize or give an ether of the composition

$$\begin{array}{ccc} CH_2 & \!\!-O- & CH_2 \\ | & & | \\ CH_2 & \!\!-O- & CH_2 \end{array}$$

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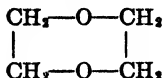
[1] Bergmann, M., and Ludewig, S., *Ann. Chem.*, 1924, cdxxxvi, 173. Bergmann, M., and Kann, E., *Ann. Chem.*, 1924, cdxxxviii, 278.

[2] Karrer, P., Polymere Kohlenhydrate, Leipsic, 1925.

[3] Pictet, A., and Ross, J. H., *Compt. rend. Acad.*, 1922, clxxiv, 1113; *Helv. Chim. Acta*, 1922, v, 876.

[4] Pringsheim, H., Die Polysaccharide, Berlin, 1923; *Naturwissenschaften*, 1925, xiii, 1084.

or more complex ones as

$$
\begin{array}{c}
\quad\ \ CH_2\!-\!CH_2\!-\!O\!-\!CH_2\!-\!CH_2 \\
O\diagdown\diagup\qquad\qquad\qquad\qquad\diagdown\diagup O \\
\quad\ \ CH_2\!-\!CH_2\!-\!O\!-\!CH_2\!-\!CH_2
\end{array}
$$
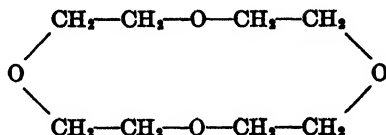
The ethers do not differ in their elementary composition from the polymers.

Which are the factors that determine the direction of the reaction towards polymerization or towards condensation? The present work is undertaken to obtain some light towards the solution of this problem.

The substances taken for the present investigation were propylene oxide and glycidol. These two substances differ from each other only in that the first contains in position (3) a methyl group, the second, in the same place, a hydroxymethyl group.

It was desired to study first those reactions which would take place under the influence of higher temperature and pressure only. In the case of propylene oxide, however, there was tested also the tendency towards condensation of propylene oxide with propylene glycol and also the condensation under the influence of a minimal quantity of a 50 per cent solution of alkali.

Experiments on condensation of propylene oxide with propylene glycol were made in the absence of catalysts and also in the presence of a trace of concentrated sulfuric acid.

In the case of the glycidol, the condensations were brought about without the action of catalysts. It must be added here that the property of glycidol to condense with itself has been observed before. The product of condensation, however, was never studied.

Experiments with Propylene Oxide.—On heating the oxide in a sealed tube at 165–167°C. for 4 weeks only a small part of the substance reacted; the greater part, about two-thirds, remained unchanged. The product which boiled at a higher temperature, namely at 122–135°C., had the odor and other properties of the diether of propylene glycol.

$$
\begin{array}{c}
CH_2\!-\!O\!-\!CH_2 \\
\mid\qquad\quad\ \ \mid \\
CH_3\!\cdot\!CH\!-\!O\!-\!CH\!\cdot\!CH_3
\end{array}
$$
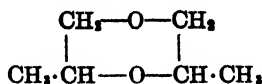
For identification the ether was prepared by distillation of propylene glycol with sulfuric acid. The substance obtained under such conditions was definitely the diether and not a polymer of propylene oxide for the reason that it was prepared on distillation of the di(hydroxypropyl) ether,

$$
\begin{array}{ccc}
CH_2\!-\!O\!-\!CH_2 & & \\
| & | & \\
CH_3\ CHOH & CHOHCH_3 &
\end{array}
$$
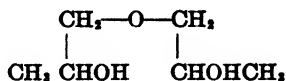
The di(hydroxypropyl) ether was obtained from propylene oxide on heating with very little alkali (0.5 cc. of a 50 per cent solution to 8.5 gm. of the oxide). Undoubtedly, part of the oxide was hydrolyzed to the glycol and the reaction proceeded in the following way.

$$
\begin{array}{ccccc}
CH_2 & HOCH_2 & & CH_2\!-\!O\!-\!CH_2 \\
| \quad \diagdown & | & & | \qquad | \\
\quad \quad O + & & \rightarrow & \\
| \quad \diagup & | & & | \qquad | \\
CH_3CH & CHOHCH_3 & & CH_3\ CHOH \quad CHOHCH_3
\end{array}
$$
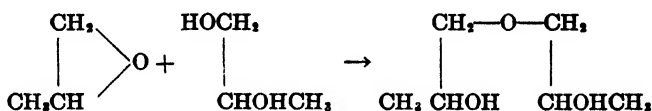
Indeed, the same substance was obtained on condensation of propylene oxide with propylene glycol in the absence of catalysts, or in the presence of a trace of sulfuric acid.

As regards the condensation of propylene oxide in the presence of a little alkali, it may be added that the reaction does not stop at the dimolecular ether, inasmuch as alongside with the dihydroxy ether, substances were obtained which analyzed well for the trimolecular and for the tetramolecular complexes.

Thus, under the conditions thus far employed, propylene oxide showed great reactivity towards the formation of condensation products. Polymerization in the true sense, has as yet not been observed. This, however, does not mean that conditions for polymerization will not be found in the future. Efforts to accomplish this end will be continued in this laboratory.

Experiments with Glyc·dol.[5]—On heating anhydrous glycidol for 42 hours, at the temperature of the boiling steam bath, a product was obtained which distilled at 128–130°C. at 1.5 mm. and had the same elementary composition as glycidol. The sub-

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[5] *Cf.* Bresslauer, M., *J. prakt. Chem.*, 1879, xx, 188.

stance, thus, could be either a product of polymerization of the glycidol or a condensation product of the following composition.

$$CH_2OH \quad CH_2$$
$$| \qquad\qquad | \quad\rangle O$$
$$CHOH \quad CH$$
$$| \qquad\qquad |$$
$$CH_2-O-CH_2$$
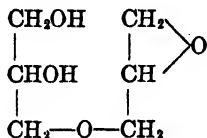
In order to decide between the two structures, the substance was hydrolyzed with dilute sulfuric acid and the product obtained in this manner analyzed not for glycerol, as it should have in case of simple polymerization, but for the following substance.

$$CH_2OHCHOHCH_2$$
$$\rangle O$$
$$CH_2OHCHOHCH_2$$
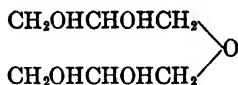
Thus the condensation undoubtedly proceeded in the following way.

$$CH_2 \qquad\quad HOCH_2 \qquad\quad CH_2-O-CH_2$$
$$| \ \rangle O \qquad | \qquad\qquad | \qquad\qquad |$$
$$CH \qquad + \quad CH \qquad\quad CHOH \quad CH$$
$$| \qquad\qquad | \ \rangle O \rightarrow | \qquad\qquad | \ \rangle O$$
$$CH_2OH \qquad CH_2 \qquad\quad CH_2OH \quad CH_2$$
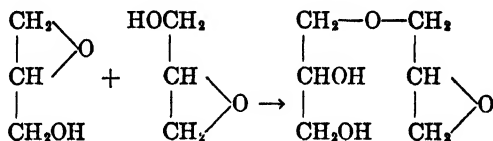
On heating at 125°C. for several days, a higher condensation product was obtained.

The elementary composition of the substance was identical with that of glycidol but it was a heavy, viscous substance, non-distillable and possessing the molecular weight of a 6-molecular glycidol. On hydrolysis with dilute sulfuric acid a distillable substance could not be obtained from it.   On treatment with ammonia a substance was obtained which contained only a small quantity of amino nitrogen.   On account of these properties and because of the results of the condensation experiment at lower temperature, it is assumed that the substance consists of one or several condensation products of the general formula

$$CH_2OHCHOHCH_2 \mid O\ CH_2\ CHOH\ CH_2 \mid n-OCH_2-CH\overset{\displaystyle O}{\overbrace{\qquad}}CH_2$$

Distillation of Propylene Glycol in the Presence of Sulfuric Acid.—
To 40 gm. of propylene glycol were added 1.1 cc. of concentrated
sulfuric acid (sp. gr. = 1.84).   The solution was distilled.   The
distillate consisted of two layers; the bottom layer weighed 15
gm. and was colorless; the top layer weighed 21.5 gm. and had a
greenish color.

The top layer was taken up in ether, washed, with dilute sodium
carbonate until it was free from acid, and then with water until
the solution gave only a faint test for aldehyde with fuchsin and
sulfurous acid, as well as with sodium nitroprusside and piperidine.
To remove the traces of the aldehydes the solution was allowed to
stand overnight with Tollens' reagent.   The reaction product was
extracted with ether.   The ethereal solution was washed, dried
over sodium sulfate, and distilled.   The presence of traces of alde-
hydes even after a repeated treatment with Tollens' reagent
suggests that some aldehyde is continually produced by hydrolysis
of the propionic aldehyde propylene glycol acetal which very
probably formed from the diether of propylene glycol.

The residue after removing the ether was

I.   114–117° about 8.5 cc.
II.   127–170°   "   2   "   at 771 mm. pressure.

The first fraction was refractionated in a small distilling flask
with a column into the following parts.

I.   112–114°, 2   cc.
II.   114–117°, 4.5 "
III.   117–125°, 1   "
Residue 0.3 cc.

The analysis of the third fraction corresponds to the propylene
glycol diether

$$CH_3—CH—CH_2$$

$$CH_3—CH—CH_3$$

or,

$$CH_3 \: CH \underline{\quad\quad} CH_2$$
$$| \qquad /$$
$$O \quad O$$
$$|/$$
$$CH\underline{\quad}CH_2\underline{\quad}CH_3$$
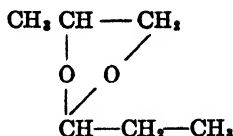
0.0934 gm. substance: 0.2125 gm. CO_2 and 0.0881 gm. H_2O.
$C_6H_{12}O_2$. Calculated. C 62.07, H 10.34.
Found.   " 62.04, " 10.55.

On distilling 2.9 gm. of di(hydroxypropyl) ether (preparation described later in this paper) in the presense of 0.06 cc. of concentrated sulfuric acid between 80–115°C., 2 cc. of a mixture were obtained consisting of 1.5 cc. of a yellow top layer and 0.5 cc. of an aqueous bottom layer.   In the distilling flask remained a black residue.   The aqueous part gave an aldehyde reaction with a sulfurous fuchsin solution.   The top layer was taken up in ether to which a little ice was added.   The mixture was neutralized with sodium carbonate solution and washed until neutral (the aqueous wash water still gave the aldehyde reaction).   The ether solution was dried over sodium sulfate, then a little sodium was added to remove traces of water, and the ether was removed by distillation with a long column.   From the residue a fraction was received boiling from 110–119°C. at ordinary pressure.   This substance represents dimethyldioxane.

2.683 mg. substance: 6.110 mg. CO_2 and 2.604 mg. H_2O.
$C_6H_{12}O_2$. Calculated. C 62.07, H 10.34.
Found.   " 62.10, " 10.77.

Condensation of Propylene Oxide.—8 gm. of propylene oxide (not entirely anhydrous) were kept in a sealed tube at 165–167°C. for 4 weeks.   Two-thirds of the reaction mixture distilled over at the temperature of unchanged propylene oxide.   1 cc. of substance distilled between 122–135°.   The odor of the latter fraction was similar to that obtained on distilling propylene glycol or isohydroxypropyl ether in the presence of sulfuric acid. It represents very probably impure dimethyldioxane.   A small amount of higher boiling liquids was obtained boiling from 110–140°C. and 140–170°C. at 5 mm. pressure.   These two liquids

represent a mixture of various polyethers which are described below.

Action of Potassium Hydroxide on Propylene Oxide.—8.5 gm. of optically active propylene oxide ($[\alpha]_D^{20} = +10.3°$) to which 0.5 cc. of a 50 per cent potassium hydroxide solution had been added, were heated at 117–118°C. for 12 days. The reaction product was subjected to fractional distillation. A small fraction, 1.5 cc., distilled at 128–130°C. and 2.4 mm. pressure. The temperature of the bath was 167–170°C.

A second fraction (about 2 cc.) was obtained at 130–140°C. and 2.4 mm. pressure, the temperature of the bath being 175–185°C. This liquid showed a slight turbidity. The greater part could not be distilled. In the distilling flask remained 3.5 gm. of a brownish viscous liquid.

The analysis of the first fraction corresponds to a propylene glycol triether. It is as follows:

5.386 mg. substance: 11.071 mg. CO_2 and 5.155 mg. H_2O.
$C_9H_{20}O_4$. Calculated.   C 56.19, H 10.50.
              Found.   " 56.05, " 10.72.

The rotation of the substance in water was

$$\frac{-5.46° \times 100}{1 \times 12.69} = -43.0°.$$

The second fraction analyzed for a tetramolecular condensation product.

4.154 mg. substance: 8.834 mg. CO_2 and 3.987 mg. H_2O.
$C_{12}H_{26}O_5$. Calculated.   C 57.55, H 10.47.
              Found.   " 57.99, " 10.74.

The rotation of the substance in water was

$$[\alpha]_D^{20} = \frac{-6.05° \times 100}{1 \times 12.30} = -49.19°.$$

Action of Propylene Glycol on Propylene Oxide.—5 gm. of propylene glycol and 8 gm. of propylene oxide were heated at a temperature of 117–118°C. for 12 days. The following fractions were obtained.

   I. Fraction boiling from 90–103°C. at 5 mm. pressure, yielding about 2 cc.

  II. Fraction boiling from 99–100°C. at 2 mm. pressure, yielding about 2 cc.

III. Fraction boiling from 93–103°C. at 0.8 mm. pressure, yielding about 4 cc.

The residue was further separated into two fractions.

    IV. Fraction boiling from 120–125°C. at 1.6 mm. pressure.
    V.   "   "   "   126–131°C. " 1.6 "   "

The temperature of the bath during the last fractionation was 162–168°C.

The analysis for the second and third fraction was as follows:

5.895 mg. substance: 11.586 mg. CO_2 and 5.553 mg. H_2O.
2.488 "   "   : 4.935 "   "   " 2.433   "   "
$C_6H_{14}O_3$. Isohydroxypropyl ether. Calculated.   C 53.69, H 10.52.
                                         Found. II. " 53.59, " 10.54.
                                         III. " 54.09, " 10.94.

The analysis of the first fraction indicates that it also consists mostly of the di(hydroxypropyl) ether contaminated with a little unchanged propylene glycol.

The analyses of the fourth and fifth fractions correspond to a substance of the following composition.

$$CH_2\text{—}O\text{—}CH_2$$
$$|\qquad\qquad|$$
$$CH(OH)CH_2\text{—}O\text{—}CH\qquad CHOH$$
$$|\qquad\qquad|\qquad\quad|$$
$$CH_3\qquad\qquad CH_3\quad CH_3$$

The formation of this compound is very probably due to the interreaction of 1 molecule of propylene oxide with 1 molecule of the di(hydroxypropyl) ether. This substance analyzed as follows:

3.109 mg. substance: 6.420 mg. CO_2 and 3.001 mg. H_2O.
5.887 "   "   : 12.146 "   "   " 5.441 "   "
      $C_9H_{20}O_4$. Calculated.   C 56.19, H 10.50.
                 Found. IV. " 56.26, " 10.34.
                       V. " 56.31, " 10.80.

In the following experiment 4 gm. of optically active propylene oxide ($[\alpha]_D^{20} = +10.3°$) and 2.6 gm. of optically active propylene

glycol were heated at 117–119°C. for 8 days. About 1 gm. of unchanged propylene oxide and 2.5 gm. of propylene glycol were recovered. The fraction boiling from 95–100°C. at 4 mm. pressure was redistilled and a fraction received at 129–132°C. at 30 mm. pressure. This fraction represents optically active di(hydroxypropyl) ether.

The analysis was as follows:

3.944 mg. substance: 7.630 mg. CO_2 and 3.743 mg. H_2O.
$C_6H_{14}O_3$.   Calculated.   C 53.69, H 10.52.
            Found.   " 52.75, " 10.61.

The rotation of the substance in water was

$$[\alpha]_D^{20} = \frac{-3.00° \times 100}{1 \times 8.24} = -36.4°.$$

Condensation in Presence of Sulfuric Acid.—To 3.8 gm. of optically active propylene glycol ($[\alpha]_D^{20} = -14°$) were added 2.9 gm. of inactive propylene oxide and 0.02 cc. of concentrated sulfuric acid. A reaction took place immediately and spontaneously. After the initial reaction subsided, the mixture was heated for 6 hours on a steam bath under a reflux. After 15 hours standing, 12 cc. of water were added and the mixture was neutralized with a barium hydroxide solution. After heating with a little charcoal and filtering, the solvent was removed by distillation under reduced pressure. Some unchanged propylene glycol was recovered. The fraction boiling from 115–130°C. at 19 mm. pressure was redistilled at ordinary pressure (765 mm.) boiling from 220–235°C. (uncorrected). It represents di(hydroxypropyl) ether. The analysis was as follows:

3.404 mg. substance: 6.687 mg. CO_2 and 3.229 mg. H_2O.
$C_6H_{14}O_3$.   Calculated.   C 53.69, H 10.52.
            Found.   " 53.59, " 10 61.

The rotation in water was

$$[\alpha]_D^{20} = \frac{-1.98° \times 100}{1 \times 10.02} = -19.76°.$$

The higher boiling fractions were not analyzed.

Condensation of Glycidol.—The condensation of glycidol pro-

ceeds at ordinary temperature very slowly if at all, at 100°C. moderately, and at 160°C., glycidol turns, within a few hours, very viscous and difficultly soluble in ether.

30 gm. of glycidol were heated for 42 hours on a steam bath under a reflux condenser with a calcium chloride tube. The unchanged glycidol was recovered by distillation under reduced pressure boiling from 42–44°C. at 2.6 mm. Then a fraction of 3.5 gm. was obtained which boiled at 135°C. and 1.6 mm., the temperature of the bath being 170–185°C. This substance analyzed for a dimeric glycidol.

3.844 mg. substance: 6.768 mg. CO_2 and 2.941 mg. H_2O.
$C_3H_6O_2$. Calculated. C 48.61, H 8.10.
Found. " 48.01, " 8.56.

In order to test the structure of this substance, two experiments were performed.

1. It was hydrolyzed with dilute sulfuric acid. If the substance were a dimeric glycidol, the product of hydrolysis should be glycerol; otherwise it should be an ether.

2. The addition of ammonia was tested. The proportion of amino nitrogen should permit the conclusion as to the number of oxidic rings that were in the molecule.

1. 2.5 gm. of this substance were dissolved in 50 cc. of a 5 per cent sulfuric acid solution and refluxed for 5 hours. The solution was neutralized with a saturated barium hydroxide solution and a little barium carbonate. After heating with a little charcoal and filtering, the water was removed under diminished pressure, and the residue repeatedly dissolved in a little absolute alcohol and evaporated. Then the residue was distilled at 1.7 to 2 mm. pressure and a fraction received boiling from 199–202°C. The temperature of the bath was 235–250°C. The substance weighed 1.5 gm. The boiling point as well as the analysis indicates that the substance is diglycerol,

$$CH_2OHCHOHCH_2-O-CH_2CHOHCH_2OH$$
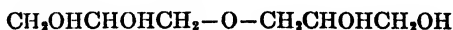
The analysis was as follows:

4.239 mg. substance: 6.755 mg. CO_2 and 3.286 mg. H_2O.
$C_6H_{14}O_5$. Calculated. C 43.35, H 8.49.
Found. " 43.45, " 8.67.

2. 1 gm. of the substance was dissolved in 200 cc. of concentrated ammonia and allowed to stand for 15 hours at 12°C. On evaporation under reduced pressure a residue was obtained which contained a considerable amount of amino nitrogen but not quite enough even for 1 nitrogen atom in the molecule. Thus, the composition of the original substance may be expressed by the following structural formula.

$$CH_2OHCHOHCH_2—O—CH_2—CH \overset{\displaystyle O}{\overbrace{\qquad}} CH_2$$
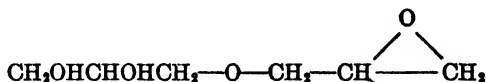
Higher Condensation Product of Glycidol.—On heating glycidol for 7 days at 125°C. in a sealed tube, a yellow non-distillable substance was obtained. The analysis agreed with a polymeric glycidol and was as follows:

0.0832 gm. substance: 0.1472 gm. CO_2 and 0.0616 gm. H_2O.
$C_3H_6O_2$. Calculated. C 48.61, H 8.10.
Found. " 48.24, " 8.28.

5 gm. of this substance were dissolved in 25 cc. of a 2 per cent sulfuric acid solution and refluxed for $7\frac{1}{2}$ hours. The solution was neutralized with barium carbonate, filtered, and the water removed under reduced pressure. The residue could not be distilled at 3 mm. pressure.

That the original substance still contained an α-oxidic ring system could be shown by dissolving 1 gm. of the substance in 200 cc. of concentrated ammonia. After 15 hours standing, the ammonia with the water was removed under reduced pressure. The residue showed some amino nitrogen, but very little. The molecular weight determination shows that 6 molecules cf glycidol had combined.

The Molecular Weight Determination by the Freezing Method.
18.9700 gm. H_2O, 0.4981 gm. substance, 0.11°C. difference in freezing temperature.

$$M = \frac{100 \times 0.4981 \times 18.5}{0.11° \times 18.970} = 441.6.$$

$6 \times C_3H_6O_2$. Calculated 444. This estimation is given in a preliminary way and will be repeated.

SUMMARY.

1. On distillation of propylene glycol in the presence of sulfuric acid the following substance was obtained.

$$CH_3—CH—CH_2$$
$$\qquad\quad |\qquad |$$
$$\qquad\quad O\qquad O$$
$$\qquad\quad |\qquad |$$
$$CH_3—CH—CH_2$$
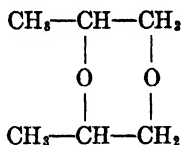
2. On condensation of propylene oxide in the absence of catalysts, the same substance is formed.

3. On condensation of propylene oxide in the presence of alkali, di(hydroxypropyl) ether is obtained, which can then be converted into the above diether.

4. Under the same conditions higher condensation products also are obtained of the type

$$CH_3\ CHOH\ CH_2—O—CH_2\ CH—O—CH_2\ CHOH\ CH_3$$
$$\qquad\qquad\qquad\qquad\qquad\qquad |$$
$$\qquad\qquad\qquad\qquad\qquad\quad CH_3$$
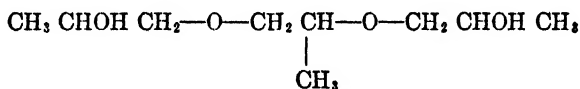
5. On condensation of propylene glycol with propylene oxide in the presence of sulfuric acid the same products are obtained as in (3) and (4).

6. Glycidol on warming at about 100°C. condenses into the substance

$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad O$$
$$\qquad\qquad\qquad\qquad\qquad\qquad / \ \backslash$$
$$CH_2OH\ CHOH\ CH_2—OCH_2—CH———CH_2$$
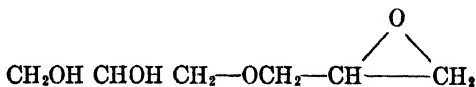
7. Glycidol on condensation at higher temperature gives higher condensation products.

ON WALDEN INVERSION.

X. ON THE OXIDATION OF 2-THIOLCARBOXYLIC ACIDS TO THE CORRESPONDING SULFONIC ACIDS AND ON THE WALDEN INVERSION IN THE SERIES OF 2-HYDROXYCARBOXYLIC ACIDS.

By P. A. LEVENE, T. MORI, and L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

In a series of 2-hydroxy acids of known configurations, such as lactic, tartaric, glyceric, and hexonic acids, it was found that those acids which had the configuration of levo-lactic acid showed a change in rotation toward the right on passing from the unionized state (free acid) to the ionized state (salt). These acids were designated as d acids. 2-hydroxy acids configurationally related to dextro-lactic acid suffered under identical conditions a change in rotation towards the left; they were designated as l acids. The rule was then applied to other 2-hydroxy acids, such as 2-hydroxybutyric, 2-hydroxyvaleric, etc., for which the configurations could not be revealed by the direct chemical method. Recently Levene and Haller and Levene and Walti found a way of elucidating by direct chemical methods the configurations of 2-hydroxybutyric, 3-hydroxybutyric, and 4-hydroxyvaleric acids and they also showed that the above rule holds for all of them. Thus, as the direct chemical investigation is extended, the indirect physical method is continually confirmed and now there seems to be little doubt that the rule holds for all hydroxy acids.

The question then arose whether the rule applied also to acids substituted in position (2) by polar groups other than -OH, such as halogens, $-NH_2$, $-SH$, $-SO_3H$, etc. For the amino derivatives Levene[1] has shown that there is reason to believe that the rule is applicable, inasmuch as it holds for 2-aminogluconic and 2-ami-

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[1] For references, see Levene, P. A., *Chem. Rev.*, 1925, ii, 179.

nomannonic acids, the configurations of which were established by other means.

For acids substituted by other groups or elements there exist no direct methods by which they could be correlated with lactic acid as the reference substance. It was therefore concluded to test whether acids substituted in position (2) by either -SH or by -SO_3H and having identical configurations show an analogous change in rotation when passing from the undissociated state into

TABLE I.

		Amino. (1)	Hydroxy. (2)	Halide. (3)	Thiol. (4)	Sulfo. (5)	Series. (6)
Propionic acid.	Free.	+13.60	+1.80	+37.96	+58.98	+13.76	
	Salt.	+2.40	−11.88	−9.34	−5.58	−3.28	*l*
n-Butyric acid.	Free.		+2.40	+20.57	+21.19	+6.66	
	Salt.		−9.55	+2.09	−1.98	0	*l*
n-Valeric acid.	Free.		+1.65	+36.72	+20.05	−0.96	
	Salt.		−3.71	+14.76	−0.69	−19.5	*l*
Isovaleric acid.	Free.	+44.21	−11.44	+8.16	+9.17	+3.04	
	Salt.	+7.48	−16.52	+3.27	+3.49	−8.07	*l*
n-Caproic acid.	Free.	+35.18	+4.95	+44.11	+23.61	−6.04	
	Salt.	+5.89	−14.76	+15.67	+1.29	−34.63	*l*
Isocaproic acid.	Free.	+26 30	−0.70	+52.22	+22.30	−8.27	
	Salt.	−13.62	−4.33	+22.52	−1.40	−39.72	*l*
Phenylacetic acid.	Free.	+295.6	+237.12	−224.9	−120.1	−3.09	
	Salt.	+170.0	+194.88	−243.1	−149.9	−14.56	*l*

the dissociated. Levene and Mikeska[2] applied the test to two 2-substituted acids; namely, to the derivatives of propionic and succinic acids, and found that the change in polarity of the substituting group did not affect the sense of the change in rotation between the undissociated acids and their ions.

It was then considered desirable to extend the observations to a

[2] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1925, lxiii, 85; 1926, lxx, 365.

TABLE II.

Acid.	Reagent.	Product.	Walden inversion.
Propionic acid.			
Dextro-lactic (*l*)......	PBr$_5$	Dextro-bromopropionic (*l*).[3]	—
" "......	SOCl$_2$	Dextro-chloropropionic (*l*).[3]	—
Dextro-chloropropionic (*l*)..........	Ag$_2$O	Levo-lactic (*d*).[4]	+
Dextro-bromopropionic (*l*).........	KOH	Dextro-lactic (*l*).[4]	—
Dextro-alanine (*l*)....	HNO$_2$	Levo-lactic (*d*).[5]	+
" "....	NOBr	Levo-bromopropionic (*d*).[6]	+
Levo-bromopropionic (*d*)...............	NH$_3$	Levo-alanine (*d*).[6]	—
Dextro-bromopropionic (*l*)..........	ROCSSK	Levo-thiolpropionic (*d*).[7]	+
n-Butyric acid.			
Dextro-bromobutyric (*l*)...............	ROCSSK	Levo-thiolbutyric (*d*).*	+
n-Valeric acid.			
Dextro-bromovaleric (*l*)...............	ROCSSK	Levo-thiolvaleric (*d*).*	+
Dextro-bromovaleric (*l*)...............	KOH	Levo-hydroxyvaleric (*d*).*	+
Isovaleric acid.			
Dextro-bromoisovaleric (*l*)...........	KOH or Ag$_2$O	Levo-hydroxyisovaleric (*d*).[8]	+
Dextro-bromoisovaleric (*l*)...........	KSH	Levo-thiolisovaleric (*d*).*	+

* Reported in this paper.
[3] Walden, P., *Ber. chem. Ges.*, 1895, xxviii, 1293.
[4] Purdie, T., and Williamson, S., *J. Chem. Soc.*, 1896, lxix, 820, 829, 837.
[5] Fischer, E., and Skito, A., *Z. physiol. Chem.*, 1901, xxxiii, 190.
[6] Fischer, E., and Warburg, O., *Ann. Chem.*, 1905, cccxl, 171.
[7] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1924, lx, 1.
[8] Fischer, E., and Scheibler, H., *Ber. chem. Ges.*, 1908, xli, 889, 2891.

TABLE II—*Continued.*

Acid.	Reagent.	Product.	Walden inversion.
Dextro-bromoisova- leric (*l*)............	NH₃	Levo-valine (*d*).[8]	+
Levo-valine† (*d*)......	HNO₂	Dextro-hydroxyisova- leric (*l*).[8]	+
" "........	NOBr	Dextro-bromoisovaleric (*l*).[8]	+
Dextro-bromoisova- leric (*l*)............	NH₃	Levo-valine (*d*).[8]	+
n-Caproic acid.			
Dextro-bromocaproic (*l*)................	KOH	Levo-hydroxycaproic (*d*).*	+
Levo-bromocaproic (*d*)................	KSH	Dextro-thiolcaproic (*l*).*	+
Isocaproic acid.			
Dextro-bromoisoca- proic (*l*)...........	KOH	Dextro-hydroxyisoca- proic (*d*).	+
Levo-bromoisocaproic (*d*)................	KSH	Dextro-thiolisocaproic (*l*).	+
Levo-bromoisocaproic (*d*)................	NH₃	Levo-leucine (*d*).[9]	−
Dextro-leucine‡ (*i*)...	HNO₂	Levo-hydroxyisoca- proic (*l*).[10]	−
" ".....	NOBr	Levo-bromoisocaproic (*d*).[11]	+
Levo-leucine ester (*l*).	NOBr	Dextro-bromoisoca- proic (*l*).[10]	−
Phenylacetic acid.			
Levo-mandelic (*d*)....	PCl₅	Dextro-chlorophenyl- acetic (*d*).[12]	−

† "Levo-valine" is designated as the substance with $[\alpha]_D^{20} = -28.7°$ in HCl, − 6.1° in H₂O.

‡ "Dextro-leucine" is designated as the substance with $[\alpha]_D^{20} = +15.8°$ in HCl, −10° in H₂O.

[9] Fischer, E., and Carl, H., *Ber. chem. Ges.*, 1906, xxxix, 3998.

[10] Scheibler, H., and Wheeler, A. S., *Ber. chem. Ges.*, 1911, xliv, 2684.

[11] Fischer, E., and Schoeller, W., *Ann. Chem.*, 1907, ccclvii, 13.

[12] Walden, P., *Ber. chem. Ges.*, 1895, xxviii, 1295.

TABLE II—*Concluded.*

Acid.	Reagent.	Product.	Walden inversion.
Levo-chlorophenyl-acetic (*l*)	NaOH	Levo-mandelic (*d*).[13]	+
Levo-chlorophenyl-acetic (*l*)	Ag₂CO₃	Dextro-mandelic (*l*).[13]	−
Dextro-aminophenyl-acetic (*l*)	HNO₂	Levo-mandelic (*d*).[14]	+
Dextro-aminophenyl-acetic (*l*)	NOCl	Levo-chlorophenyl-acetic (*l*).[15]	−
Levo-bromophenyl-acetic (*l*)	KSH	Dextro-thiophenyl-acetic (*d*).*	+

larger group of substances. The present communication is a report of the observations on derivatives of the following acids, all substituted in position (2): *n*-butyric, *n*-valeric, isovaleric, *n*-caproic, isocaproic, and phenylacetic acids. The results are summarized in Table I, which contains also the older observations on substituted propionic acids. Whenever known, the behavior of amino acids also is added.

From Table I, the following deductions may be made. First, in every sulfo acid the change in the direction of rotation on passing from the unionized to the ionized state is the same as in the corresponding thiol acid. Thus the change in the polarity of the significant group has no effect on the sense of the change in rotation and therefore this behavior may be made the basis for correlating the configurations of carboxylic acids substituted in position (2) by groups of different polarity (see Columns 4 and 5 of Table I).

Second, by making use of this principle for correlating the configurations of the substituted acids, the classification may be arranged as given in Table I. According to this classification it is seen that: (1) In propionic acid, all substituted acids having the same configuration rotate in the same direction. This observa-

[13] McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1908, xciii, 811.

[14] Fischer, E., and Weichhold, O., *Ber. chem. Ges.*, 1908, xli, 1293.

[15] McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1909, xcv, 792.

tion applies also to butyric acid.   (2) In *n*-valeric acid, the amino, hydroxy, chloro, and thiol derivatives rotate in the same direction, while the sulfo derivative rotates in the opposite direction.   In isovaleric acid, all derivatives rotate in the same direction.   (3) In *n*-caproic acid, the relationships are the same as in *n*-valeric acid, whereas in isocaproic acid, the amino, bromo, and thiol derivatives rotate in one direction, but the hydroxy and sulfo derivatives rotate in the opposite direction.   (4) In phenylacetic acid, the bromo, thiol, and sulfo derivatives rotate in the same direction, whereas the amino and hydroxy derivatives rotate in the opposite direction.

The substances given in Table I all belong to the *l* series.

This knowledge of the configuration of each derivative enables us to ascertain those reactions of substitution which are accompanied by Walden inversion.   In Table II are summarized the results of some of the reactions on acids substituted in position (2); the occurrence or non-occurrence of Walden inversion is indicated in the last column.

<center>EXPERIMENTAL.</center>

<center>*Derivatives of n-Butyric Acid.*</center>

Resolution of α-Bromo-n-Butyric Acid.—100 gm. of α-bromo-*n*-butyric acid were dissolved in 750 cc. of dry acetone.   To this solution were then added 235 gm. of carefully dried brucine.   On cooling, the brucine salt separated in crystalline form.   This salt was filtered off and extracted twice with warm acetone.   The salt was then decomposed with about one-fifth its weight of 1 : 3 hydrochloric acid and extracted with ether.   The extract was dried over sodium sulfate, the ether was removed, and the residue was distilled under reduced pressure (15 mm.).   The acid distilled at 105–107°C. and showed a rotation of

$$[\alpha]_D^{20} = \frac{+\ 7.18° \times 100}{1 \times 20.39} = +\ 35.20°. \quad [M]_D^{20} = +\ 58.8° \text{ (in ether)}.$$

$$[\alpha]_D^{20} = \frac{+\ 1.23° \times 100}{2 \times 4.74} = +\ 12.9°. \quad [M]_D^{20} = +\ 21.6° \text{ (in water)}.$$

To determine the optical activity of the salt, 0.763 gm. of the

acid was treated with 1 mol of sodium hydroxide (4.37 cc. of 2 N NaOH) and diluted to 10 cc. with water.

$$[\alpha]_D^{20} = \frac{+\,0.29° \times 100}{2 \times 8.63} = +\,1.67°. \quad [M]_D^{20} = +\,3.15°.$$

α-Thiol-n-Butyric Acid.—15 gm. of α-bromo-*n*-butyric acid with an optical rotation of

$$[\alpha]_D^{20} = \frac{+\,2.24° \times 100}{1 \times 17.41} = +\,12.86°, \; [M]_D^{20} = +\,21° \text{ (in water)}$$

were treated with an excess of a concentrated aqueous solution of potassium hydrogen sulfide. The latter was prepared from a solution of 10 gm. of potassium hydroxide in 6 cc. of water by cooling the solution thoroughly and saturating with hydrogen sulfide. The bromobutyric acid was added to the sulfide solution slowly with thorough cooling. The reaction was allowed to proceed overnight at room temperature and was finally brought to completion by heating for $\frac{1}{2}$ hour on the steam bath. Hydrochloric acid was then added and the thiol acid extracted with ether, dried over sodium sulfate, and finally distilled under reduced pressure (15 mm.). It distilled at 103–107°C. and showed an optical rotation of

$$[\alpha]_D^{20} = \frac{-\,2.18° \times 100}{1 \times 12.34} = -17.66°. \; [M]_D^{20} = -\,21.2° \text{ (in ether)}.$$

To determine the rotation of the monosodium salt, 0.520 gm. of the acid was treated with 4.33 cc. of N sodium hydroxide, and the solution diluted to 10 cc. This corresponds to 0.614 gm. of monosodium salt.

$$[\alpha]_D^{20} = \frac{+\,0.17° \times 100}{2 \times 6.14} = +\,1.38°. \quad [M]_D^{20} = +\,1.96°.$$

In a similar way the rotation of the dipotassium salt was determined by treating 0.537 gm. of the acid with 2 mols of N potassium hydroxide and making up the volume to 5 cc. This corresponds to 0.878 gm. of the dipotassium salt.

$$[\alpha]_D^{20} = \frac{+\,0.11° \times 100}{2 \times 17.5} = +\,0.31°. \quad [M]_D^{20} = +\,0.61°.$$

The free acid analyzed as follows:

0.1254 gm. substance: 0.2468 gm. BaSO₄.
$C_4H_8O_2S$. Calculated. S 26.72. Found. S 27.04.

Levo-α-Sulfo-n-Butyric Acid.—10 gm. of levo-α-thiol-*n*-butyric acid

$$[\alpha]_D^{20} = \frac{-1.21° \times 100}{1 \times 6.26} = -19.3°, \; [M]_D^{20} = -23.2° \text{ (in ether)}$$

were dissolved in water and cooled to 0°C. Bromine was then added slowly until it was no longer decolorized. The solution was then evaporated to dryness under reduced pressure, the residue was dissolved in water, and the solution neutralized with potassium carbonate. The solution was again evaporated almost to dryness and absolute alcohol was then added. An oil precipitated which crystallized on stirring. This process was repeated. The salt was then precipitated from the aqueous solution with alcohol in the form of snow-white needle-like crystals.

The dipotassium salt was found to be completely inactive.

For the free acid,

$$[\alpha]_D^{20} = \frac{-1.08° \times 100}{2 \times 13.59} = -3.97°. \quad [M]_D^{20} = -6.66° \text{ (in } H_2O).$$

For the monopotassium salt,

$$[\alpha]_D^{20} = \frac{-0.28 \times 100}{2 \times 6.69} = -2.09°. \quad [M]_D^{20} = -4.31°.$$

The dipotassium salt analyzed as follows:

0.1035 gm. substance: 0.0720 gm. K₂SO₄ (for K).
0.1200 "   "   : 0.1116 "   BaSO₄ (" S).
$C_4H_6O_6SK_2$. Calculated. K 32.02, S 13.10.
                Found.   " 31.21, " 13.35.

Derivatives of n-Valeric Acid.

Resolution of α-Bromo-n-Valeric Acid.—In preliminary experiments on the resolution of α-bromo-*n*-valeric acid by means of brucine, cinchonidine, strychnine, and quinine, the best results

were obtained with quinine. A warm solution of 100 gm. of the bromo acid in 600 cc. of acetone was treated with 179 gm. of quinine. On cooling, the salt separated in the form of white needle-like crystals. It was recrystallized several times from acetone. To decompose the quinine salt it was dissolved with cooling in a slight excess of dilute hydrochloric acid and the solution was extracted with ether. The ethereal extract was washed with water and dried over sodium sulfate. The ether was then removed and the residue fractionated under reduced pressure. The substance distilled at 123–124°C. (p = 15 mm.), and showed an optical activity of

$$[\alpha]_D^{20} \quad \frac{+2.22° \times 100}{1 \times 7.16} \quad +31.0° \text{ (in ether)}.$$

$$[\alpha]_D^{20} = \frac{+0.56° \times 100}{2 \times 1.38} = +20.3°. \quad [M]_D^{20} = +36.7° \text{ (in water)}.$$

30 gm. of the active acid were obtained.

To determine the activity of the sodium salt, 0.3865 gm. of the bromo acid were treated with 1 equivalent of cold N sodium hydroxide and the solution was made up to 5 cc. The solution therefore contained 0.865 gm. of the salt per 10 cc.

For the salt,

$$[\alpha]_D^{20} = \frac{+1.26° \times 100}{2 \times 8.66} = +7.28°. \quad [M]_D^{20} = +14.76°.$$

The substance analyzed as follows:

0.0988 gm. substance: 0.1026 gm. AgBr.
    $C_5H_9O_2Br$. Calculated. Br 44.20. Found. Br 44.19.

Levo-α-Xantho-n-Valeric Acid.—29 gm. of dextro-α-bromo-*n*-valeric acid ($[\alpha]_D^{20} = +26.66°$ in ether) were suspended in 145 cc. of water, cooled to −5°C., and neutralized with 11 gm. of potassium carbonate. While the solution was still cold, 26 gm. of potassium xanthate were added. The mixture was allowed to stand at 0°C. for 3 days. 30 cc. of concentrated hydrochloric acid were then added and the solution was extracted with ether. The ethereal extract was washed with water and dried over sodium sulfate. On removal of the ether, a sticky mass was obtained, which without further purification was used for the preparation of

α-thiol-n-valeric acid.   The yield of xantho derivative was 45 gm.   Its optical activity in ether solution was

$$[\alpha]_D^{20} = \frac{-\ 0.69° \times\ 100}{1 \times 3.80} = -\ 18.1°.$$

Levo-α-Thiol-n-Valeric Acid.—45 gm. of levo-α-xantho-n-valeric acid ($[\alpha]_D^{20} = -18.1°$) dissolved in 450 cc. of absolute alcohol and thoroughly cooled, were treated with 135 cc. of cold concentrated aqueous ammonia.   The solution was allowed to stand at 0°C. for 5 days.   Most of the solvent was then removed under reduced pressure and the residue was rendered alkaline with ammonia and extracted with ether to remove the thioxanthogen amide.   The greater part of the excess of ammonia was removed from the residue by concentration under reduced pressure and the residue was then acidified with concentrated hydrochloric acid and extracted several times with ether.   The combined ethereal extracts were washed with water and dried over sodium sulfate.   The ether was removed at low temperature and the residue was fractionated under reduced pressure (about 13 mm.).   The thiol acid boiled at 122–124°C.   12 gm. of the thiol acid were obtained from 29 gm. of the bromo derivative, corresponding to 55 per cent of the theoretical yield.   It rotated polarized light to the left.

$$[\alpha]_D^{20} = \frac{-\ 0.99° \times\ 100}{1 \times 5.79} = -\ 17.1°\ \text{(in ether)}.$$

$$\frac{}{1 \times 7.09} \qquad -\ 14.8°. \quad [M]_D^{20} = -\ 19.8°\ \text{(in 60 per cent alcohol)}.$$

The optical activity of the mono- and disodium salts was determined as follows: 1.069 gm. of the thiol acid were treated with 1 equivalent of 2 N sodium hydroxide and the volume was made up to 5 cc.   This corresponds to 2.489 gm. of the mono-salt per 10 cc.

For the monosodium salt

$$[\alpha]_D^{20} = \frac{+\ 0.11° \times\ 100}{1 \times 24.8} = +\ 0.44°. \quad [M]_D^{20} = +\ 0.69°.$$

Another equivalent of 2 N sodium hydroxide was added and the volume was made up to 10 cc.   This corresponds to 1.422 gm. of the di-salt per 10 cc.

For the disodium salt,

$$[\alpha]_D^{20} = \frac{+ 1.01^\circ \times 100}{2 \times 14.2} = + 3.55^\circ. \quad [M]_D^{20} = + 6.32^\circ.$$

The free acid analyzed as follows:

0.1310 gm. substance: 0.2254 gm. BaSO$_4$.
$\quad$ C$_5$H$_{10}$O$_2$S. Calculated. S 23.88. Found. S 23.64.

Dextro-α-Sulfo-n-Valeric Acid.—4 gm. of levo-α-thiol-n-valeric acid ($[\alpha]_D^{20} = -17.1^\circ$ in ether) were suspended in 20 cc. of water and the suspension was cooled. 74 cc. (1 equivalent) of 0.4 N barium hydroxide were added to dissolve the thiol acid. The solution was buffered with 35 gm. of barium carbonate (6 equivalents) and treated with 14 gm. of bromine (6 equivalents) in small portions. The excess of barium carbonate was filtered off and the filtrate was rendered neutral to litmus with barium hydroxide. It was then evaporated to dryness under reduced pressure. The residue was taken up in hot water, and alcohol was then added to the hot solution, whereupon the barium sulfonate precipitated as a white amorphous substance. The salt was redissolved and reprecipitated with alcohol. This operation was repeated until the salt thus obtained was free from bromides. 2.000 gm. of the salt were dissolved in 15 per cent hydrochloric acid and the volume made up to 10 cc.

For the free acid,

$$[\alpha]_D^{20} = \frac{+ 0.22^\circ \times 100}{2 \times 11.4} = + 0.96^\circ.$$

The barium salt was difficultly soluble in water and was therefore converted to the sodium salt to observe the optical activity of the ion. 4.300 gm. of another crop of barium salt were shaken with an equivalent amount of sodium sulfate solution for 1.5 hours at room temperature. The mixture was then centrifuged and the barium sulfate washed thoroughly with water. The original decantate and the washings were combined and diluted to 20 cc. This corresponds to 1.533 gm. of disodium salt per 10 cc. of solution. Hence for the disodium salt,

$$[\alpha]_D^{20} = \frac{+ 2.70^\circ \times 100}{2 \times 15.33} = + 8.81^\circ. \quad [M]_D^{20} = + 19.5^\circ.$$

To determine the activity of the acid salt, the above solution was treated with 1 equivalent of hydrochloric acid. The volume was 23 cc. This corresponds to 1.203 gm. of the monosodium salt per 10 cc. Hence, for the monosodium salt,

$$[\alpha]_D^{20} = \frac{+\,0.20° \times 100}{4 \times 12.0} = +\,0.42°. \quad [M]_D^{20} = +\,0.86°.$$

For the free acid, another equivalent of hydrochloric acid was added and the volume was made up to 25 cc. This corresponds to 0.987 gm. of free acid per 10 cc. Hence,

$$[\alpha]_D^{20} = \frac{+\,0.21° \times 100}{4 \times 9.87} = +\,0.53°. \quad [M]_D^{20} = +\,0.96°.$$

The barium salt analyzed as follows:

0.0956 gm. substance: 0.0712 gm. BaSO₄ (for Ba).
0.1301 "   "   : 0.0924 "   "   (" S).
$C_5H_8O_5SBa$.   Calculated.   Ba 43.22, S 10.09.
                  Found.   " 43.83, " 9.75.

Derivatives of Isovaleric Acid.

Resolution of α-Bromoisovaleric Acid.—Berlingzzi and his co-worker[16] have recently resolved this acid by means of brucine but by this method it is somewhat difficult to prepare the acid in quantity. Cinchonidine was found more convenient for this purpose.

50 gm. of the inactive bromo acid were dissolved in 350 cc. of acetone and 82 gm. of pure cinchonidine were then added. After cooling, the salt was filtered and recrystallized from acetone nine times. It was decomposed with a slight excess of dilute hydrochloric acid and extracted with ether. The ethereal extract was washed with water and dried over sodium sulfate. On removal of the ether, the bromo acid was obtained in crystalline form. It was purified by fractional distillation under reduced pressure. The fraction distilling at 119–120°C. (p = 14 mm.) showed an optical rotation of

$$[\alpha]_D^{20} = \frac{-\,0.50° \times 100}{1 \times 6.46} = -\,7.7° \text{ (in ether)}.$$

[16] Berlingzzi, S., and Furia, M., *Gazz. chim. ital.*, 1926, lvi, 828.

The mother liquor of the above salt was allowed to stand overnight at 0°C. and another crop of crystals was obtained.   The filtrate from these crystals was concentrated under reduced pressure and the residue decomposed as usual.   The bromo acid thus obtained showed a rotation of

$$[\alpha]_D^{20} = \frac{+\ 0.73° \times 100}{1 \times 9.82} = +\ 7.43°\ \text{(in ether)}.$$

Another sample which had the optical activity of $[\alpha]_D^{20} = \dfrac{+0.54° \times 100}{1 \times 7.12} = +7.6°$ in ether, showed the following rotation in water.

$$[\alpha]_D^{20} = \frac{+\ 0.14° \times 100}{2 \times 1.55} = +\ 4.5°.\quad [M]_D^{20} = +\ 8.1°.$$

The optical rotation of the sodium salt was determined as follows: 1.407 gm. of the acid were treated with 1 equivalent of sodium hydroxide and the volume was made up to 5 cc.   This corresponds to 2.815 gm. of sodium salt per 10 cc.

$$[\alpha]_D^{20} = \frac{+\ 0.81° \times 100}{2 \times 28.15} = +\ 1.44°.\quad [M]_D^{20} = +\ 2.92°.$$

The free acid analyzed as follows:

0.1072 gm. substance: 0.1100 gm. AgBr.
    $C_5H_9O_2Br$.   Calculated.   Br 44.20.   Found.   Br 43.67.

Dextro-α-Thiolisovaleric   Acid.—α-Bromoisovaleric   acid   was treated with potassium xanthate for 3 days at room temperature in aqueous solution.   In another experiment this reaction was tried in alcohol.   In neither case was a positive result obtained. Potassium hydrogen sulfide was then·used according to the directions of Duvillier.[17]   In his paper no experimental details are given.

10 gm. of levo-α-bromoisovaleric acid ($[\alpha]_D^{20} = -7.7°$ in ether) were added under cooling to a potassium hydrogen sulfide solution prepared by saturating a solution of 8 gm. of potassium hydroxide

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[17] Duvillier, M. E., *Comp. rend. Acad.*, 1878, lxxxvi, 49; *Bull. Soc. chim.*, 1878, xxx, series 2, 507.

in 5 cc. of water with hydrogen sulfide (5 gm.) under cooling. The mixture of bromo acid and potassium hydrogen sulfide was allowed to stand overnight at 0°C. and then heated for 30 minutes on the steam bath to complete the reaction.   After cooling it was acidified with concentrated hydrochloric acid, whereupon the thiol acid separated as a semisolid.   This was extracted with ether and the ether extract was washed with water and dried over anhydrous sodium sulfate.   On removal of the ether, the residue solidified.   It was fractionated under reduced pressure and the thiol acid distilled at 114–115°C. (about 13 mm.).   It had the following rotation in ether.

$$[\alpha]_D^{20} = \frac{+\ 3.23° \times 100}{2 \times 12.1} = +\ 13.3°.$$

The active acid, as well as the racemic compound, was solid at room temperature.   (In the literature the compound is described as a liquid.)   It melted at about 35°C.   The yield was 6 gm.; *i.e.*, 90 per cent of the theory.

Resolution of α-Thiolisovaleric Acid.—This acid was resolved into its optical antipodes because the resolution of the corresponding bromo acid was more difficult.   25 gm. of the inactive thiol acid, which was prepared by heating the bromo acid and potassium hydrogen sulfide for 3 hours on the steam bath (not allowed to stand at 0°C.), were dissolved in 250 cc. of acetone.   55 gm. of cinchonidine were then added.   The salt thus obtained was extracted with acetone (filtered at room temperature) eight times. It was decomposed with an excess of dilute hydrochloric acid and extracted with ether.   The ethereal extract was washed with water and dried over sodium sulfate.   The residue from the ether was fractionated under reduced pressure.   The optical rotations were as follows:

$$[\alpha]_D^{20} = \frac{+\ 0.44° \times 100}{1 \times 3.21} = +\ 13.7°\ \text{(in ether)}.$$

$$[\alpha]_D^{20} = \frac{+\ 0.50° \times 100}{1 \times 7.31} = +\ 6.83°. \quad [M]_D^{20} = +\ 9.2°\ \text{(in 40 per cent alcohol)}.$$

For the determination of the rotations of the mono- and disodium salts, 1.064 gm. of the above thiol acid were treated with 1

equivalent of sodium hydroxide and the volume was made up to 5 cc. This corresponds to 2.477 gm. of mono-salt per 10 cc. Hence,

$$[\alpha]_D^{20} = \frac{+1.11° \times 100}{2 \times 24.7} = +2.24°. \quad [M]_D^{20} = +3.49°.$$

To the above solution 1 more equivalent of sodium hydroxide was added and the mixture was diluted to 10 cc. This corresponds to 1.413 gm. of disodium salt per 10 cc. Hence, for the di-salt,

$$[\alpha]_D^{20} = \frac{-1.14° \times 100}{2 \times 14.13} = -4.03°. \quad [M]_D^{20} = -7.17°.$$

The free acid analyzed as follows:

0.1084 gm. substance: 0.1960 gm. BaSO$_4$.
$C_5H_{10}O_2S$. Calculated. S 23.88. Found. S 24.84.

Dextro-α-Sulfoisovaleric Acid.—2 gm. of dextro-α-thiolisovaleric acid ($[\alpha]_D^{20} = +13.3°$ in ether) were dissolved in 40 cc. of a saturated solution of barium hydroxide under cooling and 15 gm. of barium carbonate were added. Bromine was then added drop by drop until it was no longer consumed. The total bromine used was 7.5 gm. The solution was neutralized to litmus with barium hydroxide and filtered from the excess of barium carbonate. To the clear filtrate some alcohol was added, whereupon the barium salt precipitated as a white amorphous substance. This was redissolved in hot water (somewhat difficultly soluble) and reprecipitated with alcohol. This operation was repeated once more. It had a rotation in 10 per cent hydrochloric acid of

$$[\alpha]_D^{20} = \frac{+0.51° \times 100}{2 \times 14.11} = +1.80°.$$

The rotations of neutral and acid salts and of the free acid were determined as follows: 1.669 gm. of barium salt were shaken for 1½ hours at room temperature with an equivalent of sodium sulfate solution. The mixture was centrifuged and the barium sulfate washed thoroughly with water. The decantate and the washings were combined and the volume was made up to 10 cc.

This corresponds to 1.19 gm. of disodium salt.   Hence, for the di-salt,

$$[\alpha]_D^{20} = \frac{- 0.85° \times 100}{2 \times 11.9} = - 3.57°.   [M]_D^{20} = - 7.92°.$$

To the above solution 1 equivalent of hydrochloric acid was added and the mixture was diluted to 15 cc.   This corresponds to 7.16 gm. of mono-salt per 10 cc. of solution.

$$[\alpha]_D^{20} = \frac{+ 0.14° \times 100}{2 \times 7.16} = + 0.98°.   [M]_D^{20} = + 2.02°.$$

Another equivalent of hydrochloric acid was added and the volume was made up to 20 cc.   This corresponds to 4.79 gm. of free sulfonic acid per 10 cc.

$$[\alpha]_D^{20} = \frac{+ 0.16° \times 100}{2 \times 4.79} = + 1.67°.   [M]_D^{20} = + 3.04°.$$

The barium salt analyzed as follows:

0.0952 gm. substance: 0.0684 gm. BaSO$_4$ (for Ba).
0.1152 "   "   : 0.0848 "   "   (" S).
        $C_5H_8O_5SBa$.   Calculated.   Ba 43.22, S 10.09.
                Found.   " 42.28, " 10.11.

Derivatives of n-Caproic Acid.

Resolution of α-Bromo-n-Caproic Acid.—60 gm. of inactive bromo acid were dissolved in 500 cc. of hot acetone and 102 gm. of pure strychnine were then added, whereupon the strychnine salt crystallized immediately.   The salt was extracted with hot acetone seven times.   It was dissolved in well cooled, concentrated hydrochloric acid and extracted with ether.   The ethereal extract was washed with ice water until the washings contained no halogen ion and dried with sodium sulfate.   The ether was distilled off and the residue fractionated under reduced pressure.   The bromo acid boiled at 129–130°C. (p = 14 mm.).   It had optical activities as follows:

$$[\alpha]_D^{20} = \frac{- 1.27° \times 100}{1 \times 4.71} = - 27.0° \text{ (in ether).}$$

$$[\alpha]_D^{20} = \frac{-0.57° \times 100}{2 \times 1.26} = -22.6°. \quad [M]_D^{20} = -44.1° \text{ (in 30 per cent alcohol).}$$

0.3140 gm. of the same substance was neutralized with sodium hydroxide and the volume was made up to 5 cc. This corresponds to 0.699 gm. of salt per 10 cc. Hence, for the sodium salt,

$$[\alpha]_D^{20} = \frac{-1.01° \times 100}{2 \times 6.99} = -7.22°. \quad [M]_D^{20} = -15.67°.$$

The substance analyzed as follows:

0.1028 gm. substance: 0.1002 gm. AgBr.
$C_6H_{11}O_2Br$. Calculated. Br 41.03. Found. Br 41.48.

Dextro-α-Thiol-n-Caproic Acid.—5 gm. of levo-α-bromo-n-caproic acid ($[\alpha]_D^{20} = -27.0°$ in ether) were added slowly to the solution of potassium hydrogen sulfide prepared from 7 gm. of potassium hydroxide and 4 gm. of hydrogen sulfide. The mixture was allowed to stand for 16 hours at 0°C. and was then heated on the steam bath for 30 minutes. After cooling, it was acidified with concentrated hydrochloric acid and extracted with ether. The ethereal extract was washed with water and dried over sodium sulfate. The residue from the ether was fractionated under reduced pressure. It boiled at 112–115°C. (1.8 mm.). The rotations were

$$[\alpha]_D^{20} = \frac{+1.69° \times 100}{1 \times 5.77} = +29.29° \text{ (in ether).}$$

$$[\alpha]_D^{20} = \frac{+1.11° \times 100}{2 \times 3.48} = +15.9°. \quad [M]_D^{20} = +23.5° \text{ (in 40 per cent alcohol).}$$

To determine the rotation of the monosodium salt, 0.5720 gm. of thiol acid was treated with 1 equivalent of sodium hydroxide and diluted to 5 cc. This corresponds to 1.316 gm. of mono-salt per 10 cc.

$$[\alpha]_D^{20} = \frac{+0.20° \times 100}{2 \times 13.16} = +0.76°. \quad [M]_D^{20} = +1.29°.$$

1 more equivalent of sodium hydroxide was added and the vol-

ume was made up to 10 cc.   This corresponds to 0.686 gm. of di-
sodium salt per 10 cc.   Hence, for the di-salt,

$$[\alpha]_D^{20} = \frac{-0.83° \times 100}{2 \times 6.86} = -6.05°. \quad [M]_D^{20} = -11.6°.$$

The free acid analyzed as follows:

0.1304 gm. substance: 0.2036 gm. BaSO$_4$.
$C_6H_{12}O_2S$.   Calculated.   S 21.62.   Found.   S 21.45.

Levo-α-Sulfo-n-Caproic Acid.—2.5 gm. of dextro-α-thiol-*n*-
caproic acid were dissolved in barium hydroxide solution by shak-
ing and to the solution were added 14 gm. of barium carbonate.
9 gm. of bromine were then added drop by drop under cooling
(the bromine must not be added too rapidly).   At the end of the
reaction barium sulfocapronate crystallized out.   This was dis-
solved by heating and the excess of barium carbonate was filtered
off.   On cooling, the salt crystallized out in needles.   From the
mother liquor another crop of the salt was obtained by adding
alcohol.   Both were combined and recrystallized two times by
redissolving in hot water and reprecipitating with alcohol.   The
barium salt thus obtained was difficultly soluble in water and to
observe the rotation it was converted to the sodium salt.

2.5 gm. of the barium salt were shaken for 1.5 hours at room
temperature with an equivalent amount of sodium sulfate solution.
Then the mixture was centrifuged and the barium sulfate was
washed thoroughly.   The decantate and the washings were com-
bined and made up to 10 cc.   This corresponds to 1.812 gm. of
disodium salt per 10 cc.   Hence,

$$[\alpha]_D^{20} = \frac{-5.23° \times 100}{2 \times 18.12} = -14.43°. \quad [M]_D^{20} = -34.63°.$$

To the above solution 1 equivalent of hydrochloric acid was
added and the volume was made up to 12 cc.   Hence, for the
monosodium salt,

$$[\alpha]_D^{20} = \frac{-0.76° \times 100}{2 \times 13.7} = -2.77°. \quad [M]_D^{20} = -6.04°.$$

Another equivalent of hydrochloric acid was added and the total volume was brought to 14 cc.   Hence, for the free acid,

$$[\alpha]_D^{20} = \frac{-0.65° \times 100}{2 \times 10.57} = -3.08°. \quad [M]_D^{20} = -6.04°.$$

To suppress the ionization of the acid the above solution was diluted with the same volume of concentrated hydrochloric acid and the specific rotation was as follows:

$$[\alpha]_D^{20} = \frac{-0.26° \times 100}{2 \times 5.29} = -2.46°.$$

The barium salt analyzed as follows:

0.1000 gm. substance: 0.0097 gm. H_2O.
 $C_6H_{10}O_5SBa.2H_2O$.   Calculated.   Water of crystallization 9.81.
                       Found.   "   "   "   9.70.
0.0903 gm. substance: 0.0629 gm. $BaSO_4$ (for Ba).
0.1084 "   "   : 0.0760 "   "   (" S).
          $C_6H_{10}O_5SBa$.   Calculated.   Ba 41.39, S 9.67.
                       Found.   "   40.99, " 9.63.

Derivatives of Isocaproic Acid.

Resolution of α-Bromoisocaproic Acid.—α-Bromoisocaproic acid was resolved into its optical enantiomorphs by Fischer[18] by means of brucine but in our work quinine was used for this resolution. To 50 gm. of inactive bromo acid dissolved in 400 cc. of acetone, 95 gm. of pure quinine were added.   After seven recrystallizations from acetone the salt was decomposed under cooling with a slight excess of dilute hydrochloric acid and the solution was extracted with ether.   The ethereal extract was washed with water and dried over sodium sulfate.   The ether was removed and the residue fractionated under reduced pressure.   It distilled at 131–131.5° (p = 16 mm.).   The optical activity was

$$[\alpha]_D^{20} = \frac{+1.79° \times 100}{1 \times 6.00} = +29.8° \text{ (in ether)}.$$

$$[\alpha]_D^{20} = \frac{+0.45° \times 100}{2 \times 0.84} = +26.8°. \quad [M]_D^{20} = +52.2° \text{ (in 20 per cent alcohol)}.$$

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[18] Fischer, E., and Carl, H., *Ber. chem. Ges.*, 1906, xxxix, 3996.

The rotation of the sodium salt was determined as follows: 0.481 gm. of the above acid was neutralized with 1 equivalent of 2 N sodium hydroxide and diluted to 5 cc.   This corresponds to 1.071 gm. of sodium salt per 10 cc.   Hence,

$$[\alpha]_D^{20} = \frac{+ 2.22° \times 100}{2 \times 10.71} = + 10.37°. \quad [M]_D^{20} = + 22.50°.$$

The substance analyzed as follows:

0.1018 gm. substance: 0.0996 gm. AgBr.
        $C_6H_{11}O_2Br$.   Calculated. Br 41.03.   Found. Br 41.63.

Levo-α-Thiolisocaproic Acid.—5 gm. of dextro-α-bromoisocaproic acid ($[\alpha]_D^{20} = +29.8°$ in ether) were added to a potassium hydrogen sulfide solution prepared as described above.   The mixture was allowed to stand for 2 days at 0°C. and heated for 30 minutes on the steam bath.   After cooling, it was acidified with hydrochloric acid and extracted with ether.   The ethereal extract was washed with water and dried over sodium sulfate.   On removal of the ether an almost colorless liquid was obtained which was purified by fractional distillation under reduced pressure.   The thiol acid boiled at 126–126.5°C. (p = 15 mm.).   It had a rotation in ether of

$$[\alpha]_D^{20} = \frac{- 0.50° \times 100}{1 \times 3.20} = - 15.6°.$$

The substance analyzed as follows:

0.1104 gm. substance: 0.1800 gm. BaSO₄.
        $C_6H_{12}O_2S$.   Calculated. S 21.62.   Found. S 22.40.

Resolution of α-Thiolisocaproic Acid.—The inactive thiol acid was prepared in the same way as the active substance, but in this case the mixture of bromo acid and potassium hydrogen sulfide was heated for 3 hours on the steam bath without standing at 0°C.

A warm solution of 46 gm. of the inactive thiol acid in 300 cc. of acetone was treated with 117 gm. of pure quinine.   The salt thus obtained was recrystallized four times from acetone and then decomposed as usual with dilute hydrochloric acid.   The ethereal extract was evaporated and the residue fractionated under reduced

pressure (p = 15 mm.).   The thiol acid boiled at 127–128°C. and showed the optical rotations of

$$[\alpha]_D^{20} = \frac{+1.93° \times 100}{1 \times 9.95} = +19.4° \text{ (in ether).}$$

$$[\alpha]_D^{20} = \frac{+1.42° \times 100}{2 \times 4.71} = +15.1°. \quad [M]_D^{20} = +22.3° \text{ (in 40 per cent alcohol).}$$

To determine the rotation of the monosodium salt, 0.603 gm. of the above thiol acid was treated with 1 equivalent of sodium hydroxide and diluted to 5 cc.   This corresponds to 1.387 gm. of mono-salt per 10 cc.   Hence,

$$[\alpha]_D^{20} = \frac{-0.12° \times 100}{1 \times 13.87} = -0.87°. \quad [M]_D^{20} = -1.48°.$$

For the disodium salt 1 more equivalent of sodium hydroxide was added to the above solution and the volume was made up to 10 cc.   This corresponds to 0.724 gm. of di-salt.

$$[\alpha]_D^{20} = \frac{-1.69° \times 100}{2 \times 7.236} = -11.68°. \quad [M]_D^{20} = -22.4°.$$

The free acid analyzed as follows:

0.1246 gm. substance: 0.2054 gm. BaSO$_4$.
$\quad\quad$ C$_6$H$_{12}$O$_2$S.   Calculated.   S 21.62.   Found.   S 22.65.

Levo-α-Sulfoisocaproic Acid.—2 gm. of dextro-α-thiolisocaproic acid ($[\alpha]_D^{20} = +19.4°$ in ether) were dissolved in 34 cc. of 0.4 N barium hydroxide solution with cooling and 16 gm. of barium carbonate were then added.   The mixture was oxidized with 7 gm. of bromine.   The excess of barium carbonate was filtered off and the clear filtrate was evaporated under reduced pressure.   The residue was taken up in hot water and alcohol was added slowly until the solution contained 50 per cent of alcohol, whereupon the barium sulfonate crystallized out in long needles.   Solution in hot water and precipitation with alcohol were repeated twice.

To determine the optical rotations of the di- and mono-salts and of the free acid, the barium salt was converted to the sodium salt. 2.1 gm. of barium salt were treated with an equivalent of sodium sulfate solution at room temperature.   The barium sulfate was

separated by centrifugation and washed thoroughly with water. The filtrate and washings were combined and diluted to 10 cc. This corresponds to 1.522 gm. of disodium salt per 10 cc.

$$[\alpha]_D^{20} = \frac{-5.14° \times 100}{2 \times 15.22} = -16.9°. \quad [M]_D^{20} = -40.6°.$$

To the above solution 1 equivalent of hydrochloric acid was added and the volume made up to 12 cc. This corresponds to 1.152 gm. of monosodium salt per 10 cc. Hence, for the acid salt,

$$[\alpha]_D^{20} = \frac{-0.78° \times 100}{2 \times 11.52} = -3.38°. \quad [M]_D^{20} = -7.37°.$$

Another equivalent of hydrochloric acid was added and the volume was made up to 14 cc. This corresponds to 0.888 gm. of the free acid. Hence,

$$[\alpha]_D^{20} = \frac{-0.75° \times 100}{2 \times 8.88} = -4.22°. \quad [M]_D^{20} = -8.27°.$$

The barium salt analyzed as follows:

0.1000 gm. substance: 0.0087 gm. H_2O.
$C_6H_{10}O_5SBa.2H_2O$. Calculated. Water of crystallization 9.81.
    " $1\frac{1}{2}$ "   "   " " "   7.54.
          Found.   " " "   8.70.
0.0903 gm. substance: 0.0627 gm. $BaSO_4$ (for Ba).
0.0940 "   "   : 0.0678 "   " (" S).
        $C_6H_{10}O_5SBa$. Calculated. Ba 41.39, S 9.67.
             Found.   " 40.41, " 9.96.

Derivatives of Phenylacetic Acid.

Resolution of α-Bromophenylacetic Acid.—The inactive bromo acid used in this experiment was prepared by heating mandelic acid with fuming hydrobromic acid in an autoclave.[19] 87 gm. of the inactive bromo acid were resolved by means of morphine, according to the directions of McKenzie.[20] The bromo acid thus resolved melted at 87–88°C. and gave an optical rotation of

$$[\alpha]_D^{20} = \frac{-3.16° \times 100}{1 \times 3.02} = -104.6°. \quad [M]_D^{20} = -225.0° \text{ (in ether)}.$$

[19] Glaser, R., *Z. Chem.*, 1868, 142.
[20] McKenzie, A., and Bate, S. C., *J. Chem. Soc.*, 1915, cvii, 1691.

Owing to the strong reactivity of the bromine atom of this acid, the accurate determination of the activity of the sodium salt was difficult. Even near 0°C. the rotation decreased because of hydrolysis to the hydroxy acid while the reading was being taken. 0.1985 gm. of the bromo acid was neutralized with 1 equivalent of sodium hydroxide with cooling and stirring and the solution was made up to 5 cc. All these operations were performed as quickly as possible.

$$[\alpha]_D^t = \frac{-4.48° \times 100}{1 \times 4.368} = -102.6°. \quad [M]_D^t = -243.0°.$$

The free acid analyzed as follows:

0.1124 gm. substance: 0.0990 gm. AgBr.
$\quad\quad$ $C_8H_7O_2Br$. Calculated. Br 37.24. Found. Br 37.48.

Dextro-α-Thiolphenylacetic Acid. (*Thiomandelic Acid.*)—10 gm. of levo-α-bromophenylacetic acid ($[\alpha]_D^{20} = -106.4°$) were added under cooling to 18 cc. of potassium hydrogen sulfide prepared as described above. The mixture was allowed to stand overnight at 0°C. and then heated for 30 minutes on the steam bath. It was acidified with concentrated hydrochloric acid and extracted with ether. The ethereal extract was washed with water until the washings no longer contained halogen ion. It was then dried over sodium sulfate. On removal of the ether a viscous liquid was obtained. (After several days part of it crystallized.) It showed the following rotation.

$$[\alpha]_D^{20} = \frac{+8.55° \times 100}{1 \times 7.47} = +114.5° \text{ (in ether)}.$$

When this product was fractionated under diminished pressure (1.8 mm.) only half of it distilled over at 148–152°C. From the results of analysis and the color reaction with ferric chloride, this part was identified as the thiol acid. It melted at 80–87°C. The yield was 3 gm. It had a rotation of

$$[\alpha]_D^{20} = \frac{+2.76° \times 100}{1 \times 3.86} = +71.5°. \quad [M]_D^{20} = +120.0° \text{ (in ether)}.$$

The residue from the distillation crystallized from benzene in the form of prisms. It gave no ferric chloride reaction and melted

at 127–130°C.   From the analysis it seems to us that it is α,α'-thioldiphenylacetic acid.   Its optical rotation was

$$[\alpha]_D^{20} = \frac{+\ 2.03° \times 100}{1 \times 3.78} = +\ 53.7° \text{ (in ether)}.$$

From the above results it can be easily understood that the optical rotation was much decreased by distillation.   Such a racemization has, to our knowledge, never been observed in aliphatic α-thiol acids.

To determine the rotation of the monosodium salt, 0.4100 gm. of thiol acid was treated with 1 equivalent of sodium hydroxide and diluted to 5 cc.   This corresponds to 0.9266 gm. of monosalt per 10 cc.   Hence,

$$[\alpha]_D^{20} = \frac{+\ 7.31° \times 100}{1 \times 9.266} = +\ 78.9°. \qquad [M]_D^{20} = +\ 149.9°.$$

Another equivalent of sodium hydroxide was added and the volume made up to 10 cc.   This corresponds to 0.517 gm. of disodium salt.   Hence, for the di-salt,

$$[\alpha]_D^{20} = \frac{+\ 1.72° \times 100}{1 \times 5.17} = +\ 33.2°. \qquad [M]_D^{20} = +\ 70.5°.$$

The fraction which distilled analyzed as follows:

0.1226 gm. substance: 0.1804 gm. $BaSO_4$.
$\qquad$ $C_8H_8O_2S$.   Calculated.   S 19.05.   Found.   S 20.21.

The residue from the distillation analyzed as follows:

0.1212 gm. substance: 0.0988 gm. $BaSO_4$.
$\qquad$ $C_{16}H_{14}O_4S$.   Calculated.   S 10.60.   Found.   S 11.20.

Dextro-α-Sulfophenylacetic Acid.—The oxidation of α-thiomandelic acid by means of nitric acid and potassium permanganate was attempted, but neither was satisfactory.   The oxidation with bromine in the presence of barium carbonate was also unsatisfactory, since there was no method of purifying the barium sulfonate because of its insolubility.   In this experiment the thiol acid was oxidized by bromine in 50 per cent acetic acid solution and in aqueous solution in the presence of a slight excess of sodium carbonate.   In the first case, 2 gm. of dextro-thiomandelic acid

($[\alpha]_D^{20} = +71.5°$) were dissolved in 30 cc. of glacial acetic acid, diluted with 30 cc. of water, and oxidized with 7 gm. of bromine under cooling and stirring. It was then filtered and the filtrate was evaporated almost to dryness under reduced pressure. The residue was taken up in water and extracted with ether to remove an insoluble oily substance. The aqueous layer was made neutral to litmus with sodium carbonate and again evaporated to small volume. Alcohol was then added until the solution contained 70 per cent of alcohol and this solution was gradually cooled and rubbed with a glass rod. The sodium sulfonate crystallized out, sometimes in the form of plates and sometimes of prisms. After the crystals had once been obtained, it was found preferable to seed the solution. The solution in water and precipitation with alcohol were repeated.

In the case of oxidation with sodium carbonate and bromine, 2 gm. of the same thiol acid were dissolved in 40 cc. of sodium carbonate solution (5 gm. of Na_2CO_3) and 7 gm. of bromine were then added drop by drop under cooling. The solution was neutralized to litmus with dilute hydrobromic acid and evaporated to small volume. On adding alcohol, the sodium sulfonate crystallized out while the bromide remained in solution.

The sodium salt thus obtained had the following rotation. 0.42 gm. of salt were dissolved in 5 cc. of water.

$$[\alpha]_D^{20} = \frac{+ 0.47° \times 100}{1 \times 8.4} = + 5.6°. \quad [M]_D^{20} = + 14.6°.$$

To the above solution 1 equivalent of hydrochloric acid was added and the volume was made up to 7 cc. This corresponds to 0.549 gm. of acid salt per 10 cc. Hence, for the acid salt,

$$[\alpha]_D^{20} = \frac{+ 0.20° \times 100}{2 \times 5.49} = + 1.83°. \quad [M]_D^{20} = + 4.36°.$$

1 more equivalent of hydrochloric acid was added and the volume was made up to 10 cc. This corresponds to 3.48 gm. of sulfonic acid. Hence, for the free acid,

$$[\alpha]_D^{20} = \frac{+ 0.10° \times 100}{2 \times 3.48} = + 1.43°. \quad [M]_D^{20} = + 3.09°.$$

The sodium salt analyzed as follows:

0.1030 gm. substance: 0.0168 gm. H_2O.
$C_8H_6O_5SNa_2.3H_2O$. Calculated. Water of crystallization 17.19.
   "   2 "   "   " "   "   12.16.
         Found.   " "   "   16.31.
0.0956 gm. substance: 0.0512 gm. Na_2SO_4 (for Na).
0.0862 "   "   : 0.0816 " $BaSO_4$ (" S).
    $C_8H_6O_5SNa_2$. Calculated. Na 17.69, S 12.31.
         Found.   " 17.34, " 13.00.

α-Hydroxy Acids.

Levo-α-Hydroxy-n-Valeric Acid.—5 gm. of dextro-α-bromo-*n*-valeric acid ($[\alpha]_D^{20} = +31.0°$ in ether) were added under cooling and shaking to 20 cc. of a solution containing 3.5 gm. of sodium carbonate. The mixture was heated for 5 hours on the steam bath. After cooling it was acidified with a slight excess of concentrated hydrochloric acid and extracted with ether. The ethereal extract was dried over sodium sulfate. The ether was evaporated and the residue taken up in water and made neutral to litmus with barium hydroxide solution. On adding alcohol to the hot solution, the barium salt crystallized out in the form of plates. This salt was redissolved in hot water and reprecipitated with alcohol. It had a wax-like luster. 1.4 gm. of the anhydrous barium salt were dissolved in 1 equivalent of hydrochloric acid and diluted to 5 cc. This corresponds to 1.781 gm. of free acid. Hence,

$$[\alpha]_D^{20} = \frac{-0.50° \times 100}{2 \times 17.81} = -1.40°. \quad [M]_D^{20} = -1.65°.$$

To the above solution 1 equivalent of sodium sulfate was added. The barium sulfate was filtered and washed thoroughly. To the filtrate and washings 1 equivalent of sodium hydroxide was then added and the volume was made up to 10 cc. This corresponds to 1.057 gm. of sodium salt. Hence, for the sodium salt,

$$[\alpha]_D^{20} = \frac{+0.56° \times 100}{2 \times 10.57} = +2.65°. \quad [M]_D^{20} = +3.71°.$$

The barium salt analyzed as follows:

1.000 gm. substance: 0.0023 gm. H_2O.
$\quad$ $C_{10}H_{18}O_6Ba.\frac{1}{2}H_2O$.$\quad$ Calculated.$\quad$ Water of crystallization 2.37.
$\qquad\qquad\qquad\qquad$ Found.$\qquad$ " $\quad$ " $\qquad$ " $\qquad$ 2.30.
0.0968 gm. substance: 0.0446 gm. $BaSO_4$.
$\quad$ $C_{10}H_{18}O_6Ba$.$\quad$ Calculated.$\quad$ Ba 36.99.$\quad$ Found.$\quad$ Ba 36.50.

Dextro α-Hydroxy-n-Caproic Acid.—4 gm. of levo-α-bromo-*n*-caproic acid ($[\alpha]_D^{20} = -28.5°$ in ether) were suspended in 3 cc. of water and 2.4 gm. of sodium carbonate were then added. The mixture was heated for 6 hours on the steam bath under a reflux condenser. After cooling it was acidified with concentrated hydrochloric acid and extracted with ether. The ethereal extract was dried over sodium sulfate and evaporated under reduced pressure. The residue which was partly crystalline was taken up in water and boiled with an excess of barium carbonate. To the filtrate from barium carbonate alcohol was added, whereupon the barium salt crystallized in plates. This was recrystallized from water and alcohol.

1.05 gm. of the barium salt were dissolved in 1 equivalent of hydrochloric acid and diluted to 5 cc. This corresponds to 1.390 gm. of free acid. Hence

$$[\alpha]_D^{20} = \frac{+\,0.20° \times 100}{2 \times 13.90} = +\,0.72°.$$

After the barium ion had been removed by sodium sulfate, 1 equivalent of sodium hydroxide was added and the solution was made up to 10 cc. For the sodium salt,

$$[\alpha]_D^{20} = \frac{-\,0.63° \times 100}{2 \times 7.84} = -\,4.02°.$$

The barium salt analyzed as follows:

0.0977 gm. substance: 0.0564 gm. $BaSO_4$.
$\quad$ $C_{12}H_{22}O_6Ba$.$\quad$ Calculated.$\quad$ Ba 34.38.$\quad$ Found.$\quad$ Ba 34.34.

Resolution of α-Hydroxy-n-Caproic Acid.—65 gm. of α-hydroxy-*n*-caproic acid were added to a warm solution of 145 gm. of cinchonidine in 700 cc. of chloroform. The mixture was evaporated and the salt obtained in crystalline form. After twelve recrys-

tallizations from water, the salt was dissolved in chloroform, cooled with ice, and decomposed with ammonium hydroxide. The aqueous layer was washed with chloroform to remove cinchonidine and evaporated to small volume. The residue was acidified with hydrochloric acid and extracted with ether. The ethereal extract was dried over sodium sulfate. On removal of the ether the residue was almost entirely crystalline. The crystals were filtered off from the syrup and recrystallized, first from dry ether and then from ether and petrolic ether. The pure hydroxy acid which crystallized in the form of plates was somewhat hygroscopic. It melted at 60–61°C. The optical rotation in water was

$$[\alpha]_D^{20} = \frac{-3.35° \times 100}{2 \times 44.6} = -3.75°. \quad [M]_D^{20} = -4.95°.$$

To the above solution 1 equivalent of sodium hydroxide was added and the solution was made up to 10 cc. For the sodium salt,

$$[\alpha]_D^{20} = \frac{+7.73° \times 100}{2 \times 26.17} = +14.77°. \quad [M]_D^{20} = +22.75°.$$

The substance analyzed by the micro method as follows:

3.920 mg. substance: 7.803 mg. CO_2 and 3.262 mg. H_2O.
$C_6H_{12}O_3$. Calculated. C 54.50, H 9.16.
Found.   " 54.28, " 9.31.

α-Hydroxyisocaproic Acid.—8 gm. of α-bromoisocaproic acid ($[\alpha]_D^{20} = -12.1°$ without solvent) were dissolved in 40 cc. of sodium carbonate solution containing 5 gm. of Na_2CO_3 and heated on the steam bath for 5 hours. The solution was then cooled and some insoluble oily matter was removed by means of ether. It was acidified and the hydroxy acid was extracted with ether. The barium salt was prepared in the same way as in the case of *n*-caproic acid.

1 gm. of the barium salt was treated as in the case of *n*-caproic acid. For free acid,

$$[\alpha]_D^{20} = \frac{-0.14° \times 100}{2 \times 13.2} = -0.53°. \quad [M]_D^{20} = -0.70°.$$

For the sodium salt,

$$[\alpha]_D^{20} = \frac{-0.42° \times 100}{2 \times 7.47} = -2.81°. \quad [M]_D^{20} = -4.33°.$$

The barium salt analyzed as follows:

0.1000 gm. substance: 0.0588 gm. $BaSO_4$.

$C_{12}H_{22}O_6Ba$.   Calculated.   Ba 34.38.   Found.   Ba 34.60.

THE RESPIRATORY METABOLISM FOLLOWING THE ADMINISTRATION OF VARIOUS CARBOHYDRATES.*

BY HARRY J. DEUEL, JR.†

(From the Laboratory of Clinical Metabolism, Mayo Clinic and The Mayo Foundation, Rochester, Minnesota.)

The physiologic behavior of glucose varies to a great extent from that of the disaccharides or the polysaccharides and even from that of its closely related optical isomers. Such differences have been frequently noted between the changes in the blood sugar level after the oral administration of glucose and those produced by the other carbohydrates. Likewise, when identical amounts of the different carbohydrates are administered, the effect of each one on the respiratory metabolism varies from that of the other.

The recent work of Folin and Berglund (8) has shown that the postprandial blood sugar values during the 1st hour vary with the carbohydrate ingested. When 200 gm. of glucose were taken, these investigators found hyperglycemia in normal men but not of sufficient magnitude to approach the ordinary kidney threshold value for this sugar. No glycosuria was noted. On the other hand, the administration of fructose, galactose, and lactose was much less effective than glucose in causing an elevation in the blood sugar level although when galactose was ingested in large amounts galactosuria usually resulted. Foster (9) and later Bodansky (3) have confirmed the observations of Folin and Berglund with regard to the behavior of fructose and glucose but they find that well defined hyperglycemia, greater than that occasioned by glucose, occurs after the ingestion of galactose and reaches a maximal value in the 3rd hour. The blood sugar level, according to Foster, is apparently an index of the rapidity of glycogenesis rather than a measure of the needs for the transportation from one organ to another to supply the demand for energy-yielding foodstuffs, a postulate proposed by Folin and Berglund. Bock,

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* A preliminary report of this work was given in *Proc. Soc. Exp. Biol. and Med.*, 1925, xxiii, 85.

While the present paper was in press, a paper of Cathcart and Markowitz (*J. Physiol.*, 1927, lxiii, 309) has appeared, which entirely confirms the results reported here.

† Special student at the Mayo Clinic during the summer of 1925.

Schneider, and Gilbert (2) have detected a rise in blood sugar as early as 3 minutes after the ingestion of 110 gm. of glucose in solution.

The rate of absorption from the intestinal tract suggests a possible clue to the greater hyperglycemia produced by galactose and glucose than by fructose. According to Cori, if the rate of absorption of glucose in the intestine of the rat is assumed to be 100, that of galactose is 110, while that of fructose is only 43. It is simple to harmonize these findings with the effect of these sugars on the blood sugar level. However, the results of Deuel and Chambers (7), who compared the rate of excretion of "extra glucose" in phlorhizinized dogs after the oral administration of the same amounts of these sugars, do not show any striking variations in the rate at which glucose is excreted, although the latter is the sum of several variables as the rate of absorption, the rate of conversion of the sugar to glucose, and the capacity of the kidney to excrete the latter. Wierzuchowski,[1] in the metabolism laboratory of the Mayo Clinic, found no difference in the rate of disappearance of glucose and levulose from the stomach when taken with barium, as determined by roentgenographic studies.

Johansson (12) reported a comparative study on the effects of the various sugars on the respiratory metabolism. He found twice the increase in the elimination of carbon dioxide in a man after the administration of fructose over that observed when a like quantity of glucose was taken. This, however, might as well indicate that the oxidation of fructose causes a higher respiratory quotient than does glucose rather than that fructose causes that much difference in heat production since no oxygen determinations were made. Benedict (1) reports that fructose exerts the most pronounced effect on metabolism of any of the common sugars, followed in activity by sucrose and glucose.

Lusk (16) has investigated the relative specific dynamic action caused by the feeding of 50 gm. of fructose, sucrose, glucose, galactose, and lactose to a normal dog. The average increase over the basal metabolism during the 2nd, 3rd, and 4th hours was greatest with fructose, amounting to 37 per cent over the basal metabolism. The others followed in this order: sucrose (34 per cent), glucose (30 per cent), galactose (22 per cent), and lactose (3 per cent). The respiratory quotient with the first three sugars during this period averaged unity or slightly above, while with galactose and lactose it amounted to about 0.90. The small increase with lactose may be attributed to the absence of lactase in the intestine of the adult dog with consequent slow conversion to glucose and galactose and therefore delayed absorption. In these experiments of Lusk on the specific dynamic action of various sugars, technical difficulties inherent in the operation of the respiration calorimeter have prevented the determination of the metabolic effect during the 1st hour after the ingestion. Slow absorption of lactose in the case of the dog was also indicated in the experiments of Deuel and Chambers in which it was noted that considerable

[1] Personal communication from Dr. Boothby.

"extra sugar" was still being eliminated in the phlorhizinized dog 12 hours after the ingestion of 16 gm. of lactose in distinction to the complete recovery, by that time, of the glucose originating from the same quantity of fructose, galactose, and glucose. According to the theory of Lusk, the increased metabolism after the ingestion of the sugars is due to a plethora of metabolites in the blood and tissues. Fructose furnishes the largest number of these since the triose molecules hypothetically formed when fructose is changed into glucose increase the number over what would be present were only glucose or undissociated fructose molecules present. The same reasoning applies to sucrose with the exception that, after its hydrolysis to fructose and glucose, only one-half of its molecules must necessarily pass through the triose stage.

The rapidity with which the carbohydrates become available, as judged by a study of the respiratory quotient, and their effect on the heat production in short intervals during the 1st hour after their administration have not been extensively investigated. The present study was therefore undertaken to determine the speed with which these reactions take place with the different sugars and with starch, as well as to make certain comparisons in the total specific dynamic action of these substances.

Methods.

The experiments reported here were carried out on the author, the majority during a period when the subject was on a high carbohydrate diet of about 1800 calories[2] which was practically protein-free. The remainder of the tests were made somewhat later in a period after the subject had had thyroxin and also had received a low protein diet and a much smaller proportion of carbohydrate than in the previous period.

The metabolism determinations were carried out with the open circuit type of respiration apparatus commonly used in the metabolism laboratory of the Mayo Clinic (4). The analyses of oxygen and carbon dioxide in the expired air were made in triplicate on the Haldane gas analysis apparatus. In the few cases in which the analyses did not check within 0.03 per cent of an atmosphere in all three samples, additional analyses were made. Therefore, as the experiments made were on a trained subject the respiratory quotients should be considered reliable. The non-protein respiratory

[2] A preliminary report of the latter work has been published by Deuel, H. J., Jr., Sandiford, K., Sandiford, I., and Boothby, W. M., *J. Biol. Chem.*, 1926, lxvii, p. xxiii.

quotient was calculated after the protein metabolism had been allowed for, as determined from the nitrogen content of the 24 hour urinary sample.   The basal metabolic rate obtained was compared with the normal standards established by Du Bois.   At least two determinations of the standard basal metabolism were made in the morning before the ingestion of the carbohydrate; after the ingestion of the carbohydrate the mask was tied on immediately so that the metabolism determinations could be started within 2 minutes.   As a regular routine the subject arrived at the laboratory at 7.30 a.m. after a short walk, voided, closing the 24 hour sample of urine, weighed himself, and reclined on the bed (where the subsequent tests were to be made) for approximately $\frac{1}{2}$ hour before the determinations of basal metabolism were begun. The basal determinations were of 15 minutes duration and usually showed very close agreement both in regard to the heat production and respiratory quotient.

The carbohydrate was taken in 75 gm. doses, usually dissolved in 200 cc. of water.   In all cases except with the starch, it was drunk with the subject assuming a sitting position for less than 30 seconds; this procedure probably had only a slight effect on the metabolic rate of the following period.   On the other hand, when starch was eaten, the subject arose from the bed, walked a few feet across the room to the table, sat for the period while he was eating the starch and, at the completion, immediately returned to bed.   Metabolism tests were carried out immediately in order to see how quickly an effect on the respiratory quotient might be evidenced.   It is realized that the total heat production of the first metabolism test after the starch was eaten was influenced not alone by the food but by the exercise in the period immediately before; however, the respiratory quotient would be affected very little if any by this amount of movement.

After the carbohydrate was ingested, a series of eleven or twelve metabolism determinations was performed in the following period of $4\frac{1}{2}$ or $5\frac{1}{2}$ hours, by which time the specific dynamic action had subsided and the respiratory quotient had returned to the basal level.   During the 1st hour four metabolism tests of 10 minutes duration were carried out, the first one starting 3 minutes after the ingestion of the carbohydrate.   For the following $2\frac{1}{2}$ hours, tests of 15 minutes duration were made at half hourly intervals.

The following two tests were 45 minutes apart and the final one an hour after the preceding test or 5½ hours after the ingestion of the carbohydrate.

Pfanstiehl sugars (c.p.) were used in the experiments reported here. The starch employed was the commercial corn-starch ordinarily used in cooking except that in one instance arrowroot starch was used.

<div align="center">EXPERIMENTAL.</div>

The experiments between June 24 and July 10 were conducted while the subject was taking daily a protein-free high carbohydrate (420 gm.) diet. The nitrogen elimination was decreasing for the first few days of the period but remained fairly stationary toward the end, in the neighborhood of 3 gm. for 24 hours. This fall in nitrogen metabolism occurred coincident with the decrease in the basal metabolism from -9, June 22, to -20, July 10. Additional experiments were carried out in a later period when the subject for a second time was placed on the protein-free high carbohydrate diet, a regimen immediately following one which for a week contained approximately 45 gm. of protein, 260 gm. of carbohydrate, and 175 gm. of fat. The nutritional status of the subject was therefore presumably quite different than in the earlier experiments both on account of the character of the diet and also by reason of the fact that 7 mg. of thyroxin had been administered July 21 and daily doses of 0.5 and 0.2 mg. had been given between August 3 and 13. In fact these influences are usually reflected in both the basal heat production and the basal respiratory quotients and they may account for the slower response in the respiratory quotient and smaller specific dynamic action that usually occurred in the later experiments. This difference in reaction may be due in part to a change in the quantity either of deposit glycogen or of deposit protein or of both combined, as the changes in the experimental conditions affect the reserves of both these substances.

<div align="center">*Experiments with Monosaccharides.*</div>

Two experiments each were carried out with glucose and fructose and three with galactose. The essential data are given in Table I.

TABLE I.

Effect on Heat Production and Respiratory Quotient of Ingestion of 75 Gm. of Various Monosaccharides.

Time (min)	Glucose I, June 30, 1925, Weight 81.8 kilos			Glucose II, Aug. 18, 1925, Weight 73.9 kilos			Fructose I, July 1, 1925, Weight 81.1 kilos			Fructose II, Aug. 25, 1925, Weight 73.4 kilos			Galactose I, July 7, 1925, Weight 80.7 kilos			Galactose II, July 10, 1925, Weight 80.1 kilos			Galactose III, Aug. 20, 1925, Weight 74.2 kilos		
	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase	Non-protein R.Q.	Total calories per hr.	Per cent increase
Basal.	0.825	62.4		0.709	74.0		0.785	62.9		0.806	66.8		0.870	63.3		0.819	60.1		0.792	71.8	
	0.818	63.1		0.755	74.0		0.809	63.3		0.815	68.4		0.851	61.3		0.793	62.5		0.776	70.7	
	0.791	61.8																			
Average.	0.811	62.4		0.732	74.0		0.797	63.1		0.811	67.6		0.861	62.3		0.806	61.3		0.784	71.3	
3	0.85	67.1	8	0.76	75.3	2	0.93	67.2	6	0.84	63.8	2	0.94	66.8	7	0.89	68.6	12	0.77	75.2	5
15	0.84	67.5	8	0.82	76.7	4	1.00	70.2	11	0.94	66.6	-1	0.95	65.4	5	0.99	66.7	9	0.85	76.3	7
30	0.85	66.5	7	0.83	76.3	3	0.97	67.2	8	0.93	71.6	6	0.99	69.7	12	1.00	67.4	10	0.88	75.9	6
50	0.89	69.1	11	0.85	76.1	3	1.03	68.0	8	1.00	69.3	3	1.03	66.2	6	1.02	70.3	15	0.99	75.7	6
65	0.94	67.7	8	0.90	79.5	7	0.98	68.1	8	0.90	69.5	3	0.92	67.7	9	0.95	69.6	14	1.00	75.7	6
90	0.90	69.6	12	0.87	77.8	5	1.04	70.9	12	0.97	73.1	8	1.00	71.1	14	0.96	69.7	14	0.97	80.0	12
120	0.88	72.9	17	0.94	82.0	11	1.00	73.8	17	1.00	75.9	12	1.02	72.2	16	0.98	71.1	16	0.97	79.0	11
150	0.89	71.3	14	0.85	79.9	8	0.93	72.0	14	0.95	70.0	4	0.95	70.8	14	0.96	68.5	12	0.90	73.3	3
180	0.92	71.2	14	0.87	75.3	2	0.93	69.6	10	0.91	71.6	6	0.87	64.6	4	0.90	66.5	8	0.88	74.2	4
225	0.87	66.5	7	0.71	74.4	1	0.86	64.9	3	0.74	67.9	0	0.90	63.2	1	0.89	65.1	6	0.79	68.9	-3
270	0.76	64.6	4	0.77	72.0	-3				0.81	67.1	-1	0.91	63.4	2	0.91	64.5	5	0.81	70.6	-1
330	0.78	65.6	5				0.86	64.0	1							0.83	66.5	8			

* To conserve space the various periods after the ingestion of the carbohydrates have been arranged according to the following time intervals, although the actual time may have varied by as much as 5 minutes.

The experiments with glucose were quite the opposite from what might be expected. With a sugar which is as rapidly absorbed as is glucose and which in all respects is to be regarded as the most truly physiologic of all the carbohydrates, a much more prompt rise in respiratory quotient would be anticipated. In the first experiment with glucose recorded in Table I, there is practically no noticeable effect on the respiratory quotient until 1 hour after its ingestion when the maximal quotient of 0.94 was obtained. In only two observations in the following $4\frac{1}{2}$ hours did the respiratory quotient equal or exceed 0.90. At the end of $4\frac{1}{2}$ hours the respiratory quotient had returned to the basal level obtained before the glucose was ingested. The second experiment with glucose showed similar results except that the maximal quotient of 0.94 (the only one exceeding 0.90) was much longer delayed and did not occur until 2 hours after the ingestion of glucose although it began to rise more promptly and from a lower basal level. The greatest increase in heat production in both experiments with glucose occurred at the beginning of the 3rd hour after its ingestion. The consideration of the actual amount of the specific dynamic action is discussed later.

When 75 gm. of fructose were taken, quite different results were obtained. The prompt availability of this carbohydrate is evident. In the metabolism determination carried out 3 minutes after the fructose ingestion, the respiratory quotient had increased in the first experiment from a basal level of 0.80 to 0.93 while, during the following 10 minute period, a value of 1.00 was obtained. The second experiment with fructose did not show as great a rise in the respiratory quotient immediately but the rise was much faster than with either of the tests with glucose. In the case of fructose as with glucose, the maximal increase in heat production occurred at the beginning of the 3rd hour in both experiments. At the end of $4\frac{1}{2}$ hours the respiratory quotient in one experiment had returned to that of the fasting level and the heat production in both experiments was that of the basal.

These results on the respiratory quotient after the ingestion of fructose and glucose are in harmony with unpublished experiments by Boothby and Wierzuchowski referred to later. A difference in reaction is also found in some experiments carried out by Lublin (15) who compared the effect of glucose and fructose on the

respiratory quotient of man both with and without insulin. Himwich, Rose, and Malev (11) in a recent paper have also noted that there is a marked retardation in the rate at which the respiratory quotient is elevated after the ingestion of glucose as compared with that following the ingestion of dihydroxyacetone.

The rate at which galactose becomes available approximates that of fructose. From Table I it will be noted in the second experiment with galactose that during the 10 minute period beginning 3 minutes after the ingestion of galactose, there was a rise from a basal quotient of 0.81 to one of 0.89 and that during the second 10 minute period, it increased to 0.99. A similar rapid rise is seen in the first experiment, while in the third the rapidity of the rise was slightly decreased. The difference in this last experiment may be due to the change in the nutritional state of the subject. In harmony with the results with glucose and fructose, the maximal specific dynamic action was manifest at the beginning of the 3rd hour. The increased heat production caused by galactose was completed within $4\frac{1}{2}$ hours after its ingestion in two of the three experiments carried out.

Experiments with Disaccharides.

Two experiments each were carried out with sucrose, maltose, and lactose, the results of which are given in Table II.

The results with sucrose, instead of indicating a delay before the carbohydrate becomes utilized due to the time necessary for hydrolysis, showed an increase in respiratory quotient as great in the first period as that obtained with fructose, while in the second 10 minute period, starting about 15 minutes after the ingestion of the sucrose, the quotient was unity. In fact, in the first experiment with sucrose recorded in Table II, the maximal quotient of 1.05, which occurred in the period 15 minutes after the sugar was taken, is, with the exception of one found in the second lactose experiment, the highest respiratory quotient I have obtained in any period with any of the carbohydrates reported here. In both instances the immediate increase in heat production was more rapid than with the monosaccharides, although the maximal value in the second experiment was obtained at the period beginning $2\frac{1}{2}$ hours after the ingestion of the sugar, while in the first experiment it was obtained after 30 minutes. In both cases the in-

TABLE II.

Effect on Heat Production and Respiratory Quotient of Ingestion of 75 Gm. of Various Disaccharides (and for Comparison the Effect of Equal Quantities of Glucose and Fructose).

Time.	Sucrose I. June 25, 1925. Weight, 82.4 kilos.			Sucrose II. July 2, 1925. Weight, 81.7 kilos.			Maltose I. July 8, 1925. Weight, 80.2 kilos.			Maltose II. Aug. 24, 1925. Weight, 73.8 kilos.			Lactose I. July 6, 1925. Weight, 81.2 kilos.			Lactose II. July 9, 1925. Weight, 80.2 kilos.			37.5 gm. glucose + 37.5 gm. fructose. Aug. 26, 1925. Weight, 73.2 kilos.		
	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.
min.																					
Basal.	0.765	67.6		0.838	62.0		0.831	61.3		0.783	66.4		0.831	61.0		0.839	62.2		0.812	64.3	
	0.779	67.8		0.812	61.3		0.807	62.7		0.799	67.2		0.831			0.876	64.0		0.807	63.9	
Average.	0.772	67.7		0.825	61.7		0.819	62.0		0.791	66.8		0.83	61.0		0.858	63.1		0.810	64.1	
3	0.87	77.3	14	0.94	68.9	12	0.85	66.4	7	0.83	71.1	6	0.86	66.1	8	0.82	66.3	5	0.80	63.4	-1
15	1.05	76.7	13	1.00	68.0	10	0.82	65.4	5	0.83	72.0	8	0.90	62.3	2	0.87	62.7	-1	0.89	69.4	8
30	1.02	80.7	19	1.04	63.7	11	0.83	68.6	11	0.84	72.8	9	0.94	65.3	7	1.05	66.0	5	0.98	71.8	12
50	0.91	74.6	10	0.94	67.7	10	0.92	64.4	4	0.89	67.8	1	0.95	61.0	0	0.97	66.2	5	0.96	68.2	6
65	1.00	78.3	16	0.93	68.6	11	0.88	67.9	10	0.96	69.5	4	0.86	62.9	3	0.94	69.0	9	0.99	65.8	3
90	1.00	78.6	16	0.94	69.4	12	0.89	70.1	13	0.94	72.4	8	0.94	68.6	12	0.96	68.6	9	1.00	74.4	16
120	0.95	76.2	13	0.94	72.7	18	0.95	72.0	16	0.93	70.3	5	0.93	67.2	10	0.96	67.9	8	1.04	73.8	15
150	0.90	71.2	5	0.95	72.6	18	0.91	73.1	18	0.93	73.3	10	0.96	70.4	15	0.95	70.2	11	0.99	74.3	16
180	0.86	68.0	0	0.86	66.7	8	0.95	68.4	10	0.96	71.7	7	0.97	67.3	10	1.00	70.4	12	0.89	67.0	5
225	0.81	69.5	3	0.84	62.4	1	0.98	64.7	4	0.92	66.8	0	0.97	65.2	7	0.92	64.0	1	0.80	68.7	7
270	0.74	67.7	0	0.83	63.6	3	0.82	65.7	6	0.81	69.6	4	0.80	64.1	5	0.92	64.6	2	0.81	66.5	4
330													0.77	59.9	-2	0.86	63.8	1			

* To conserve space the various periods after the ingestion of the carbohydrates have been arranged according to the following time intervals, although the actual time may have varied by as much as 5 minutes.

creased respiratory quotients and heat production returned to the basal levels after $4\frac{1}{2}$ hours.

The results with maltose were almost exactly identical with those of glucose. This is exactly what one would expect since maltose yields 2 glucose molecules on hydrolysis. In each case with maltose (Table II) the effect of the ingestion of this sugar on the respiratory quotient was not marked until almost an hour afterward, the first period to show a notable increase being that one in the last 15 minutes of the 1st hour; it was, however, definitely less than that obtained from sucrose. The maximal respiratory quotient (0.98) in the first experiment occurred almost 4 hours after the ingestion of the maltose, while the highest value (0.96) in the second experiment occurred 1 and 3 hours after the maltose was taken. The greatest specific dynamic action was tardy, occurring $2\frac{1}{2}$ hours after the ingestion of the maltose in both cases. The effect of the maltose on the heat production and respiratory quotient had been dissipated in both experiments at the end of $4\frac{1}{2}$ hours.

After the administration of 75 gm. of lactose, the rise in quotient was prompter than with maltose but somewhat slower than with sucrose. A definite rise was noted in both cases (Table II) in the period commencing 30 minutes after the taking of this sugar. The maximal quotients in the first experiment (0.97) were reached after 3 and $3\frac{3}{4}$ hours, while in the second experiment a value of 1.05 was obtained in the third 10 minute period after its ingestion, a second high peak being reached in the latter case 3 hours after its ingestion. The maximal heat production occurred in the first experiment in $2\frac{1}{2}$ hours and in the second experiment in 3 hours; this is delayed somewhat in comparison with the average time of all other sugars.

In the last experiment given in Table II is shown the effect when equal amounts of fructose and glucose are ingested in a quantity approximately equal to that obtained from the hydrolysis of 75 gm. of sucrose. When 37.5 gm. each of fructose and glucose were given, the result did not differ markedly from that when 75 gm. of fructose or sucrose were given. The experiment showed as rapid a rise in respiratory quotient as the fructose experiment carried out when the subject was in a similar condition of nutrition. It would be of interest to know whether 37.5 gm. of fructose alone

would produce this effect or whether the glucose is oxidized more rapidly when given with fructose. This experiment has not been carried out.

Experiments with Polysaccharides.

Starch is known to exert considerable effect on the heat production but it is not known how soon the effect becomes manifest. In the case of the pig, Wierzuchowski and Ling (19) have demonstrated the increase in heat production even when raw starch is fed, although these investigators used much larger quantities than were used in the present studies.

One experiment was carried out with raw corn-starch, one with cooked corn-starch which had been dried, one with cooked corn-starch pudding, and one with cooked arrowroot starch pudding. The experimental data are given in Table III.

The raw corn-starch exerted practically no effect on the rate of heat production although after a preliminary lowering of the respiratory quotient a steady increase was noted until a maximal value of 0.91 was reached in the period starting $3\frac{3}{4}$ hours after ingestion of the starch. The only period which showed a slightly increased heat production was at the beginning of the 2nd hour and the rise was so small as to be of questionable import. That the corn-starch was fairly completely digested would seem probable in view of the fact that Langworthy and Deuel (14) found that raw corn-starch in quantities as large as 250 gm. daily could be so completely digested by normal men that no trace escaped in the feces.

In both experiments in which cooked starch pudding was eaten, there was almost immediate increase in heat production which continued throughout the 1st hour, after which it fell practically to that of the basal level. It is questionable whether this rise can be entirely due to the specific dynamic effect of the starch, especially in the first period after its ingestion, because no rest period intervened. The ingestion of this large bulk of starch was accompanied by considerable nausea which persisted through a large part of the 1st hour; this may be responsible, in part at least, for the higher level of heat production during this time. The maximal respiratory quotient of 0.95 was reached with the first experiment $2\frac{1}{2}$ hours after the starch was ingested, thus reaching a higher

TABLE III.

Effect on Heat Production and Respiratory Quotient of Ingestion of 75 Gm. of a Polysaccharide (and for Comparison the Effect of Breakfast).

Time.*	Raw corn-starch. June 29, 1925. Weight, 82.0 kilos.			Cooked, dried corn-starch. July 3, 1925. Weight, 81.0 kilos.			Cooked corn-starch pudding. June 26, 1925. Weight, 82.1 kilos.			Cooked arrowroot starch pudding. Aug. 27, 1925. Weight, 73.4 kilos.			Breakfast: 123 gm. carbohydrate, 10 gm. fat, 1 gm. protein. June 24, 1925. Weight, 82.5 kilos.		
	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.	Non-protein R.Q.	Total calories per hr.	Per cent increase.
min.															
Basal.	0.818	66.3		0.807	61.2		0.803	65.6		0.774	64.8		0.769	69.9	
	0.779	66.2		0.843	62.0		0.833	66.4		0.822	66.1		0.772	69.6	
Average.	**0.799**	**66.3**	0	**0.825**	**61.6**		**0.818**	**66.0**		**0.798**	**65.5**		**0.771**	**69.8**	
3	0.80	66.5	0	0.85	68.3†	11	0.64	76.2†	15	0.83	78.2	19	1.02	83.9	20
15				0.85	62.7	2	0.80	75.9†	15						
30	0.74	64.4	−3	0.81	61.8	0	0.88	77.4‡	17	0.78	76.3	16	0.98	85.2	22
50	0.79	65.5	−1	0.84	61.9	0	0.89	75.3‡	14	0.87	73.0	11	0.94	85.2	22
65	0.81	66.5	0	0.87	62.2	1	0.89	75.2‡	14	0.90	73.1	12	1.00	87.8	26
90	0.83	67.1	1	0.87	64.2	4	0.87	68.9	4	0.88	69.1	5	0.95	87.2	25
120	0.85	69.5	5	0.91	65.4	6	0.88	66.9	1	0.89	65.8	0	0.93	83.5	20
150	0.87	67.2	1	0.85	65.0	6	0.95	69.7	6	1.00	66.8	2	0.94	78.9	13
180	0.88	65.5	−1	0.85	64.7	5	0.93	68.2	3	0.94	70.6	8	0.85	73.2	5
225	0.91	65.0	−2	0.90	64.3	4	0.85	68.1	3	0.91	68.4	4	0.84	71.9	3
270	0.85	63.0	−5	0.83	61.0	−1	0.80	65.8	0	0.83	67.2	3	0.84	71.6	3
330	0.83	65.4	−1	0.89	60.6	−2	0.80	65.9	0						

* To conserve space the various periods after the ingestion of the carbohydrates have been arranged according to the following time intervals, although the actual time may have varied by as much as 5 minutes.

† No rest period.

‡ Pauses.

level than that attained with glucose. In the second experiment the highest quotient (1.00) was reached after $2\frac{1}{2}$ hours when no specific dynamic action was evident. By the end of $4\frac{1}{2}$ hours the heat production and the respiratory quotient in both cases had returned to the preprandial level.

The decrease in respiratory quotient occurring soon after the ingestion of the starch, as shown in two of the experiments in Table III, has also been quite frequently met with in the metabolism laboratory of the Mayo Clinic following the administration of glucose, especially to patients with diabetes; it has also been noticed in some experiments (results unpublished) which were carried out on the specific dynamic action of meat. It cannot be attributed to any technical difficulty. A possible explanation for it might be that an increased flow of gastric juice, with consequent withdrawal of hydrochloric acid from the blood, would result in sufficient alkalosis to call for compensatory temporary retention of carbon dioxide in the blood and tissues. This would result in lowering of the respiratory quotient and would much more readily be noted in periods of 10 minutes duration than in longer tests. The increase in alveolar carbon dioxide which is known to occur after the ingestion of food supports this hypothesis and probably synchronizes with the lowering of the respiratory quotient.

In the experiment with cooked dried corn-starch there was no nausea. With the exception of the first period, which was influenced by the exertion of eating, no rise in heat production occurred during the 1st hour. A slight rise was noted at the end of the 2nd hour when the heat production rose from the basal of 61.6 calories to 65.4 calories but in $4\frac{1}{2}$ hours it returned to the basal level. The respiratory quotient reached a maximum of 0.91 after 2 hours but remained somewhat elevated during the rest of the period, indicating a slow but steady influx of glucose from the starch. The total extra heat production was the smallest noted in any of the experiments carried out with the carbohydrates except the raw starch.

The last experiment in Table III shows the effect on the metabolic rate when the standard breakfast of the non-protein diet was taken. This was made up of 10 gm. of fat, 123 gm. of carbohydrate, and 1 gm. of protein, from orange juice, cooked starch, sugar, lettuce, and tea. The maximal respiratory quotient of 1.02

was reached in the period starting 15 minutes after the ingestion of the food, while the greatest heat production was obtained after $1\frac{1}{2}$ hours. The metabolism and respiratory quotient had nearly returned to the basal level after $5\frac{1}{2}$ hours.

Comparative Specific Dynamic Action of Carbohydrates.

In the present experiments the total specific dynamic action of various carbohydrates has been determined for the $4\frac{3}{4}$ hours following the ingestion, by which time their influence had practically subsided. The extra heat production in these cases has been computed according to the method devised by Ito[3] by plotting on standard coordinate paper, joining these points with straight lines, and determining the area between this curve and one representing the basal heat production. The latter value was determined each morning before the ingestion of the carbohydrate and was considered constant throughout the experimental period. The extent of the area, which represents the specific dynamic action, may readily be determined with a planimeter which has previously been standardized. The reading on this instrument may then be transferred into calories by a single computation. This method of determining and expressing specific dynamic action gives a means of comparing the effects of like amounts of substances on the metabolism of animals varying widely in size, as well as the effect of varying amounts of the substance on the same individual. It gives a far better method of comparison than that commonly used, which consists in making a percentage comparison between the highest peak obtained and the basal metabolism.

In the same manner the total number of carbohydrate calories has been plotted for each metabolism test and the total carbohydrate utilized during the experimental period is determined from the respiratory quotients. By deducting from this value that which would have been utilized had no carbohydrate been ingested (as determined from the basal respiratory quotient), the extra carbohydrate metabolism which can be ascribed to the particular carbohydrate ingested is ascertained. Fig. 1 shows the curves for typical experiments and illustrates the graphic method for determining these factors. The comparative specific dynamic ac-

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[3] Personal communication from Dr. Boothby.

Non-protein 1.00
Resp. Quot.
0.90
0.80
0.70

Cal. per hour 80
70

Total Cal. 60

Carb. Cal. 50
40

Fat Cal. 30
20

Pro. Cal. 10

Sp. Dy. Act. = 31.9 Cal. = 11%

Total Carb. = 180 Cal.
Basal Carb. = 104 Cal.
Extra Carb. = 76 Cal.

Hours
75 GRAMS GLUCOSE
June 30. 1925

Fig   Metal   glu
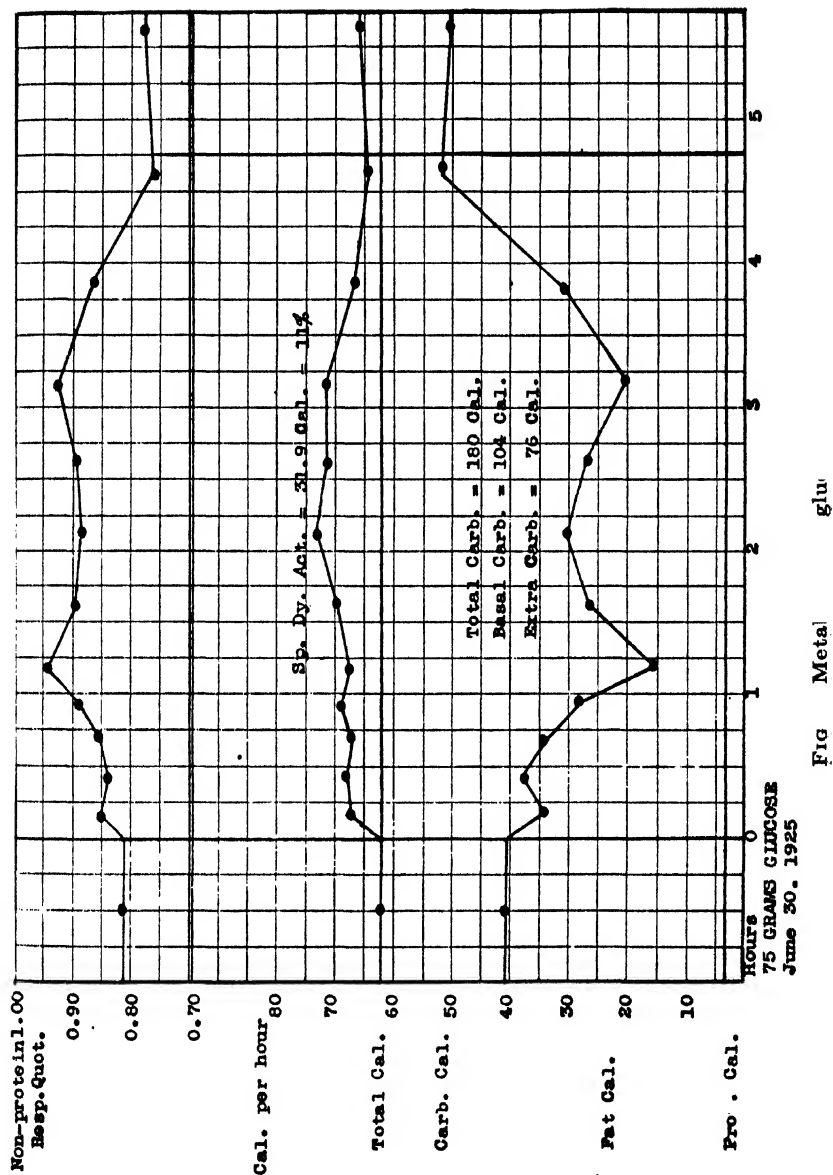
tion of the various carbohydrates for $4\frac{3}{4}$ hours after their ingestion as determined by this method is summarized in Table IV.

The maximal specific dynamic action so calculated for glucose, sucrose, galactose, and maltose is practically identical in value and equals 11 per cent of the caloric value of the carbohydrate ingested.

TABLE IV.

Relative Specific Dynamic Action of 75 Gm. of Various Carbohydrates (H J. D.).

Carbohydrate ingested.	Caloric value.	Basal R.Q.	Carbohydrate metabolized.			Specific dynamic action.	
			Total calories.	Basal calories.	Extra calories.	Calories.	Per cent of ingested carbohydrate.
Glucose......................	281	0.81	180	104	76	31.9	11
"	281	0.73	148	30	118	13.9	5
Fructose.....................	282	0.80	250	95	155	25.5	9
"	282	0.81	211	113	98	13.9	5
Galactose....................	284	0.81	236	96	140	31.7	11
"	284	0.86	245	155	90	22.6	8
"	284	0.78	206	87	119	13.1	5
Sucrose......................	297	0.83	216	114	102	31.3	11
. "	297	0.77	213	70	143	28.0	9
Maltose......................	279	0.82	213	111	102	31.3	11
"	279	0.79	221	95 .	126	18.7	7
Lactose......................	281	0.83	224	121	103	23.6	8
"	281	0.86	242	153	89	14.4	5
Glucose + fructose*...........	282	0.81	228	109	119	27.7	10*
Raw corn-starch...............	315	0.80	143	101	42	2.6	1
Cooked dried corn-starch.......	315	0.83	154	114	40	7.1	2
" corn-starch pudding....	315	0.82	173	114	59	19.4	6
" arrowroot starch pudding..................	315	0.80	203	97	106	22.9	7†

* 37.5 gm. each of fructose and glucose.

† Probably largely caused by nausea.

The maximal values for fructose (9 per cent), for lactose (8 per cent), and for cooked starch (7 per cent) are all slightly less. The average values are for sucrose 10 per cent of the caloric value of the ingested carbohydrate, for maltose 9 per cent, for glucose and galactose 8 per cent, and for fructose, lactose, and cooked starch

pudding 7 per cent. Both raw starch and cooked dried starch exerted little if any influence on the heat production, probably on account of their delayed absorption. However, it is questionable whether a part or the whole effect ascribed to starch pudding is not to be traced to the nausea.

Wierzuchowski[4] in a series of experiments carried out in the metabolism laboratory of the Mayo Clinic found quite similar results which can be summarized as follows: Levulose causes a more rapid and greater rise in the respiratory quotient than glucose and the resulting specific dynamic action from levulose amounted to

TABLE V.

Comparative Specific Dynamic Action of Glucose in Man and in Dogs.

Investigator.	Experiment No.	Subject.	Weight.	Basal metabolism.	Glucose ingested.	Duration of observation.	Specific dynamic action.
			kg.	calories per hr.	gm.	hrs.	calories
Lusk (16).	40	Dog 2.	9.3	16.19	75	5	19.80*
" (17).	35	" 3.	12.5	16.70	70	6	28.13*
" (17).	37	" 3.	12.2	16.70	70	6	31.06*
Gephart and	3–11–13	G. L.	78.4	78.60	100	3	25.94
Du Bois (10).	3–13–13	E. F. D. B.	73.6	77.64	100	4	22.46
Deuel.	Table IV.	H. J. D.	81.8	62.4	75	4.75	31.90
		H. J. D.	73.9	74.0	75	4.75	13.90

* The extra heat for the 1st hour is assumed to be equal to that of the 2nd.

6 per cent and from glucose to 3 per cent of the heat value of the carbohydrate ingested, quantities varying between 20 and 100 gm.

An analysis of the results of Lusk (17) shows that the same general increase in metabolism is caused in the dog after the ingestion of glucose as in the case of man. The results of Gephart and Du Bois (10) on men after the ingestion of 100 gm. of glucose are also of interest (Table V).

The state of nutrition probably plays an important part in the metabolism of carbohydrates so that it is difficult to make exact

[4] Personal communication from Dr. Boothby.

comparisons between the various carbohydrates studied in these experiments as well as to determine the difference in the effect produced by the same sugar in different experiments. These different effects are probably due to the continual change in the subject's nutritional condition as the result of the diet; for the greater part of the time he was on a protein free diet high in carbohydrate and at other times had small quantities of protein and less carbohydrate; in addition he was given thyroxin. In view of the differences which slight changes in nutritional state may cause, it is remarkable to find as close agreements between different experimental results as those recorded in Table V. Lusk's Dog 3, which presumably had a good reserve of glycogen, gave very close approximations on the feeding of 70 gm. of glucose (28.13 and 31.06 calories) to the results I have obtained when the subject was in good nutritional condition (31.9 calories). The results on Dog 2 were slightly lower. Gephart and Du Bois have found, as did Wierzuchowski, somewhat lower values on man than were obtained in some of my experiments.

The value for the specific dynamic action of glucose obtained in experiments on dogs and man at complete muscular rest are of the same general magnitude and suggest that in any given nutritional state the extent of the specific dynamic action does not vary with the size of the animal.

There seems to be no relationship between the amount of specific dynamic action occasioned and the degree of the total carbohydrate metabolism as shown in Table IV. When the total carbohydrate metabolism after the ingestion of lactose amounted to 242 calories, the specific dynamic action amounted to 14 calories, while with glucose, the total carbohydrate metabolism being 180 calories, the extra heat production occasioned was equal to 32 calories. Likewise, there is practically no relation between the extra carbohydrate calories caused by the ingestion of the substance under consideration and the increase in metabolism over that of the basal. For example, during the $4\frac{1}{2}$ hours after the ingestion of glucose, the carbohydrate metabolism was increased by 76 calories, while the specific dynamic action amounted to 32 calories; during a like period after the ingestion of sucrose the extra carbohydrate calories were almost double (143 calories) that

after the ingestion of glucose, while the specific dynamic action was less (28 calories). These results suggest that the extra heat production is more or less independent of the magnitude of the variations of the respiratory quotient. However, it is realized that in some cases in which very slow absorption takes place, the failure of the respiratory quotient to increase may be associated with the

TABLE VI.

Time after Ingestion of Various Carbohydrates when Maximal Respiratory Quotient and Greatest Heat Production Were Obtained (H. J. D.).

Carbohydrate ingested.	Maximal R.Q. interval.	Maximal heat production interval.
		min.
Glucose I...	65	120
" II..	120	120
Fructose I..	90 (50)	120
" II.	50 (120)	120
Galactose I.	50 (120)	120
II..	50	120
III.	65	90 (120)
Sucrose I...............	15 (30)	30 (90)
" II...............	30 (15)	150 (150)
Maltose I................	225	150
" II...............	65 (180)	150
Lactose I...............	180 (225)	150
" II...............	30 (180)	180
Glucose + fructose*.....	120	90 (150)
Raw corn-starch.........	225	120
Cooked dried corn-starch.	120	120
corn-starch pudding.......	150	150
arrowroot starch pudding.	150	180

Figures in parentheses indicate time of second high value almost equal to that of the maximum.

* 37.5 gm. each of fructose and glucose.

non-appearance of a specific dynamic effect because of this fact. Under such conditions it is conceivable that the absence of a large extra carbohydrate metabolism would be allied with a lack of extra heat production. Such a comparison may be made between an easily absorbed substance such as glucose when a small increase in respiratory quotient occurs with a high specific dynamic ac-

tivity and a material not easily absorbed such as raw starch when the same degree of increase in respiratory quotient occurs along with a practically negligible specific dynamic action.

Further analysis of the results is recorded in Table VI in which the periods of the maximal heat production and of the highest respiratory quotient are summarized. These results show that, in many cases, the maximal respiratory quotient was obtained in the 1st hour after the ingestion of the carbohydrate. For example, with sucrose, the highest respiratory quotient occurred in one experiment after 15 minutes and in the other after 30 minutes. During the 1st hour, the maximal respiratory quotients were obtained also in one of the two experiments with fructose, in all three of the experiments with galactose, and one of two experiments with lactose. With glucose the maximal quotient occurred in the 2nd hour, while the four experiments with starch and the two with maltose showed the maximal values for the respiratory quotient in the 3rd and 4th hours.

In the majority of experiments, the greatest heat production occurred at the beginning of the 3rd hour although in some cases it occurred after 150 minutes (both experiments with maltose and lactose) and in two cases after 90 minutes (one with galactose and the experiment with glucose plus fructose). In only one experiment with sucrose did it occur earlier, the period starting 30 minutes after its ingestion showing the maximal value.

There is frequently little relationship between the time of maximal heat production and that of the highest quotient. In only three experiments (Table VI) of the eighteen reported here did the highest respiratory quotient occur coincident with the greatest metabolic rate. On the other hand the plotted curves not infrequently reveal a striking parallelism between the respiratory quotient and the heat production. However, it is well to remember that the highest respiratory quotient does not necessarily mean that the greatest amount of carbohydrate is being oxidized when the oxygen absorption is constant. This fact is unquestioned when the respective respiratory quotients are above unity. For example, when the oxygen intake is constant, no greater amount of carbohydrate is being oxidized at a level of 1.15 than at one of 1.00. Even with values for the non-protein respiratory quotient somewhat below unity, we may assume that some fat is being

formed from carbohydrate according to Krogh (13). While this amount is possibly small in comparison with the total carbohydrate oxidized, the proportion may vary with identical respiratory quotients in the same animal at different times. Until better methods for determining the extent of this fat formation are available, one cannot state that the period of highest respiratory quotient is identical with the highest carbohydrate oxidation. From the experiments reported here it is certain that the greatest metabolism is not necessarily associated with the highest respiratory quotient nor does such usually occur although there is parallelism between the two curves in many instances.

The rapid rise in the respiratory quotient after the oral administration of sucrose or fructose and the retarded rate at which glucose reacts, suggest some difference in the mechanism of the reactions of fructose and glucose. To what factors then might the behavior of glucose be ascribed which would not be present in the experiments with sucrose and fructose?

In the first place one might suppose the rate of absorption would influence the speed with which a substance becomes available. Were this the only factor involved in the present experiments one should expect that fructose would be the slowest to manifest an increased respiratory quotient, since according to the results of Cori (5), it is absorbed at less than half the rate of glucose, while glucose and galactose react much more rapidly and at approximately the same rate. Such, however, is not the factor responsible in the present experiments since fructose and galactose show the most rapid effect on the respiratory quotient while the action of glucose is much retarded.

In the second place, it is hard to demonstrate any relationship between the behavior of the blood sugar level (if this also represents the tissue sugar) and the rate at which the carbohydrates are metabolized to other substances. Glucose causes the greatest rise in blood sugar during the 1st hour after its ingestion, while fructose and galactose are much less effective in this regard. One might therefore assume that the former would show a greater rise in the respiratory quotient, but this is not the case. A possible explanation is that the delay in the appearance of the metabolic reactions after the ingestion of glucose allows an accumulation of this sugar in the blood, while the greater speed with which the reactions take

place with the other monosaccharides is the cause for their lower blood sugar values.

Some differences have been noted in the behavior of these various sugars as glycogen precursors. According to Voit (18), who reported experiments on fasting rabbits and chickens, the ingestion of glucose caused the greatest increase in tissue glycogen and liver glycogen (35 gm. of glycogen for each kilo); maltose, sucrose, and fructose occupied an intermediate position (22 gm. of glycogen for each kilo); while galactose and lactose were the poorest glycogen formers (3 gm. of glycogen for each kilo). Cori and Cori (6) recently reported that glucose and fructose are on a par as glycogen agents. Even though the total glycogen formation does not vary to a great extent after the ingestion of the different sugars, it is probable that the rate of glycogenesis during the first few hours differs. Such a distinction would offer a plausible explanation for the diversity in results that I have obtained. The retarded rate or entire absence of any marked increase in respiratory quotient under these conditions of assumed low glycogen reserve would seem to offer evidence that glycogenesis is taking place. I am not aware of any experiments which have been carried out in short periods to determine the rate of glycogen formation after the ingestion of various carbohydrates. If such theories are accepted, it may be assumed that glucose, being more readily converted into glycogen, is not further metabolized until the supply offered to the tissues becomes greater than their power to retain them as glycogen. With sucrose, galactose, and fructose, the amount of sugar brought to the tissues before the glycogenetic power is exceeded may be much less, and this may explain the more rapid rise in the respiratory quotient.

SUMMARY.

The ingestion of 75 gm. of fructose or sucrose caused an almost immediate increase in respiratory quotient, the value for the 10 minute period directly following its ingestion being distinctly higher, while the second 10 minute period usually showed a respiratory quotient of unity or more. After the oral administration of galactose, the respiratory quotient showed a rapid increase at a slightly slower rate than after the ingestion of sucrose or fructose.

When glucose or maltose was taken in the same amount, a sig-

nificant rise in the respiratory quotient did not occur during the first 45 minutes. The maximal value of the respiratory quotient, which was reached in the 2nd hour with the former and in the 3rd hour with the latter, never reached unity.

With the ingestion of lactose in like quantity, a somewhat slower response was obtained than with galactose although in one case a quotient over unity was obtained in the third 10 minute period after its ingestion.

When the same amount of raw starch was ingested, there was little change in the respiratory quotient until the 2nd hour and the maximal value of 0.90 was not obtained until the 3rd hour. With cooked corn-starch pudding, the response was about the same as with glucose; if, in addition to the cooking, the starch was dried before being taken, the increase of respiratory quotient was markedly delayed, probably because of slower absorption. The highest respiratory quotient of 0.91 was not obtained until after 2 hours.

The average specific dynamic actions of the various carbohydrates were remarkably close; that for sucrose was 10 per cent of the caloric value of the ingested carbohydrates, for maltose 9 per cent, for glucose and galactose 8 per cent, and for fructose, lactose, and cooked starch 7 per cent. The variations in the individual experiments were possibly affected by differences in the nutritional condition caused by variation in diet.

The total specific dynamic action caused by the ingestion of 75 gm. of glucose in man approximates the value obtained when nearly the same quantity is fed to dogs (Lusk). This suggests that the extra heat production exerted by glucose (and probably all carbohydrates) is independent of the size of the animal which ingests it.

There is no relationship between the "extra" carbohydrate metabolism occasioned by the substance ingested and the specific dynamic action it causes. Likewise, in some instances there is no evidence of any relationship between the period in which the greatest heat production occurs and that in which the maximal respiratory quotient is found, while in other experiments the parallelism is quite striking.

The maximal heat production usually occurred 2 hours after the ingestion of the carbohydrate although with maltose and lactose it was found after 2½ hours. In one experiment with sucrose

the greatest heat production occurred 30 minutes after its inges-
tion. However, the peak of the reaction is probably of less
fundamental significance than the total specific dynamic action.

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THE SULFUR OF INSULIN.

By VINCENT du VIGNEAUD.

(From the Department of Vital Economics, the University of Rochester, Rochester, New York.)

Dudley (1) was probably the first to hint at the possibility that insulin contained sulfur, but he did not feel justified in drawing any conclusions as to the relation between activity and sulfur content. He marked the very strong organic sulfur reaction given by his purified product when boiled with lead acetate and sodium hydroxide. He was unable to obtain, however, a positive nitroprusside reaction on insulin itself or in a reduced solution of his "insulin hydrochloride." He was therefore convinced that the sulfur in the intact substance was not reducible as it is, for example, in glutathione.

Piper, Allen, and Murlin (2) and Shonle and Waldo (3) also reported a negative nitroprusside test for the sulfhydryl group. These investigators made the interesting observation that after boiling with acid the lead-blackening sulfur was reduced three-fourths.

Blatherwick (4) likewise obtained a negative test on the intact insulin. Upon reduction, however, with sodium cyanide he was able to get a very distinct positive test, showing that although the sulfhydryl group, as such, is not present in the insulin material the sulfur is present in a reducible form.

It remained for Abel and Geiling (5) to demonstrate the high degree of lability of the sulfur and its relationship to potency. They found that merely boiling with so weak an alkali as 0.1 N sodium carbonate so affected the sulfur that upon acidification hydrogen sulfide was given off. These authors felt justified from their work to draw the conclusion that the labile sulfur was directly proportional to the degree of hypoglycemic activity and that "this unstable sulfur is an integral part of the insulin molecule and that the alteration in its condition consequent upon heating with sodium carbonate bears to the destruction of the physiological activity of the hormone the relation of cause to effect."

Abel (6) has also found that his crystalline insulin contains sulfur in a labile form. In a recent publication (7) he and his collaborators have confirmed this finding although their method of obtaining the crystals is somewhat different.[1]

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[1] I have recently been able to isolate by the method devised by Abel and his coworkers a small quantity of crystals identical with those de-

Brand and Sandberg (8) have brought forth some very interesting evidence concerning the lability of the sulfur of cystine derivatives. They have shown that although the sulfur of cystine itself is quite stable, the linking of other amino acids to it has such an effect that the sulfur of the resulting compound is labile. For instance, under the same conditions of boiling with 0.1 N sodium carbonate for 45 minutes, cystine gave off only 2.8 per cent of its total sulfur, whereas dialanyl-cystine gave off 18.6 per cent and dialanyl-cystine dianhydride, 91.8 per cent. Insulin they found under the same experimental conditions gave off 35.6 per cent of the total sulfur and glutathione, 31.2 per cent. They, therefore, feel that the finding of a sulfur lability different from that of cystine for insulin cannot be interpreted as "evidence that, (a) a sulfur-containing amino acid other than cystine is present, or that (b) the sulfur in such compounds is present in more than one form of combination." Abel and Geiling's observation on the high degree of lability of insulin-sulfur is therefore not opposed to the idea that cystine is a constituent of the insulin molecule.

Brand and Sandberg tried to isolate cystine from the hydrolytic products of a sample of insulin. They obtained 150 mg. of dark brown crystals from the neutralized acid hydrolysate of 1 gm. of insulin containing approximately 21,100 units. The crystals gave a positive Millon's reaction and a very strong reaction for lead-blackening sulfur. The further purification of the material, they stated, was very difficult. From about one-half of the material they obtained some slightly yellowish crystals, which looked microscopically like cystine platelets and showed a rotation of $[\alpha]_D^{23} = -212.5°$.

Other workers have reported values for the cystine content of hydrolyzed insulin by the Folin-Looney method and by the method of Van Slyke. By the former method Shonle and Waldo (3) reported a positive reaction for cystine, and by the latter found that the cystine nitrogen was 3.2 per cent of the total nitrogen. Scott (9) found only 0.5 per cent of the total nitrogen as cystine nitrogen, and Glaser and Halpern (10) also using the Van Slyke method found none. Doisy and Weber (11), on the other hand, using the colorimetric procedure of Folin and Looney obtained a value of 13 per cent for cystine. Their product was a very pure one assaying about 40 units per mg. Blatherwick, with two products containing 25 units per mg., found by the Folin-Looney method 6.6 per cent in one and 7.1 per cent in the other. It is interesting to note that the more potent

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scribed by them. The crystals were washed with 30 cc. of ice-cold water and with 30 cc. of 95 per cent ethyl alcohol. 0.02 mg. per kilo lowered the blood sugar of a rabbit 77 mg. per 100 cc. of blood. The rabbit unitage per mg. is no doubt even higher but this happened to be the smallest amount used in the series of tests made. Slightly larger amounts caused very severe convulsions which were relieved by administration of glucose. The presence of labile sulfur in the crystals was also confirmed.

preparations contain a very high percentage of cystine as determined by the Folin-Looney method.

Neither the Folin-Looney reaction nor the Van Slyke method is specific for cystine in its application to an unknown substance. The great discrepancy between the two methods, however, is most likely explainable by the fact that boiling with strong acid so affects cystine that only 40 per cent is precipitable by phosphotungstic acid in the Van Slyke method (12). Working with small amounts of material as with insulin it is readily understandable why such low figures have been reported by this method.

That the lability of sulfur is not peculiar to insulin, but more or less a general property of proteins, is well recognized. In determining the labile sulfur of a number of proteins Blatherwick (4) found that "the labile sulfur in the insulin proteins is no more sensitive to alkalinity than the labile sulfur in keratin and zein." With gelatin and casein he obtained only a trace of sodium carbonate labile sulfur.

These workers have been led to regard the labile sulfur of insulin preparations as associated with the adsorbing proteins, and yet, they state that their biuret-free insulin contains 7 per cent labile sulfur. This is much higher than that of any of the products reported that contained protein. The observation that their biuret-free insulin contained such a large amount of labile sulfur would seem to be in favor of the view that insulin contains sulfur as an essential element. We have no evidence, however, that the disulfide linkage, *per se*, carries the physiological activity; there may well be other necessary structures present, such as the guanidine or imidazole groupings. In spite of this high sulfur content the potency of Blatherwick's biuret-free compound was not any greater per mg.; in fact, less than some of the biuret-positive samples. This might be explained in two ways. In the first place, in the process of obtaining the product, some other grouping might have been attacked, destroying some of the potency without affecting the labile sulfur. Secondly, the body of the assay animal might be able to destroy the biuret-free compound much more easily, with the result that its apparent activity is much lower than that actually present. The demonstrated greater susceptibility of the biuret-free insulin to inactivation makes this seem very likely.

Since evidence has accumulated that insulin is actually a sulfur-containing compound, it becomes imperative to discover the type of sulfur linkage. This is important as a step towards the solution of the structure of the molecule and also for establishing the minimum molecular weight from the empirical formula. It was therefore the purpose of this investigation to determine the type of sulfur linkage present and the source of the labile sulfur. It was also hoped that the study of this problem might yield a chemical method of assay.

The disulfide and the sulfhydryl types of sulfur are most common and possibly the most important linkages in biological compounds. Enough evidence existed at the time this work was undertaken to eliminate the sulfhydryl group from consideration as being present in the intact molecule. Although other types were possible, it was natural to consider first the disulfide linkage. The question immediately arose as to whether or not unhydrolyzed insulin would reduce the phosphotungstic acid reagent of Folin and Denis after preliminary reduction with sulfite, a reaction applied to the quantitative determination of cystine by Folin and Looney (13).

While many other substances reduce the phosphotungstic acid, giving a blue color, the significant fact is that most of these substances give the color directly with the reagent and do not require to be reduced first with sodium sulfite as does cystine. It can readily be seen, however, that this test cannot be considered specific for cystine, but rather for the disulfide linkage. In the field of general organic chemistry there are, at least theoretically, innumerable substances which upon reduction might in turn reduce phosphotungstic acid. In the more restricted range of biochemical compounds only cystine has been shown to act in this manner. Folin and Looney (13) state that neither tryptophane nor tyrosine, nor any other known amino acid except cystine gives the reaction in this procedure. Of sulfur compounds the disulfide linkage is the only type that will not react directly but which upon reduction with sodium sulfite in alkaline solution will give the blue color with the reagent. Such compounds as thiourea, thioacetamide, and thiobarbituric acid fail to respond to the above procedure. There is no reason to believe that sulfur linkages other than disulfide would react in the manner described for cystine.

It was found, as others have found, that insulin preparations do not give a blue color directly with the phosphotungstic reagent but upon preliminary treatment with sulfite they do react. A disturbing factor arose in the fact that upon saturation of a solution of insulin with sodium carbonate the insulin material was thrown out of solution. It was therefore decided to study the reaction on the hydrolyzed material. Later a method was found to apply the reaction to the unhydrolyzed insulin.

Source and Purification of Insulin Used.

The insulin used in the following studies was samples of puri-fied iletin U-440. 110 cc., containing 48,000 clinical units, by the manufacturer's assay, were purified by Abel's procedure (5). Sample PK represents Fraction III in this procedure, the por-tion insoluble in $N/6$ acetic acid after treatment with phenol. It was a dark brown powder weighing about 0.2 gm. Fraction IV, the portion soluble in $N/6$ acetic acid after phenol treatment, was further purified by pyridine precipitations, by salting out, and by a modification of Abel's brucine method.. Upon the addition of a $N/6$ solution of brucine to a solution of Fraction IV in $N/6$ acetic acid, a precipitate is formed. Addition of $N/6$ pyridine to the supernatant fluid causes a further precipitate to form. The bru-cine precipitate can be redissolved in $N/6$ acetic acid and repre-cipitated by the addition of brucine. Again a precipitate is formed upon the addition of pyridine to the supernatant fluid. This process of dissolving the brucine precipitate in acetic acid, reprecipitating with brucine, and then adding pyridine to the supernatant fluid was carried out until the addition of pyridine no longer gave a precipitate with the supernatant fluid. This frac-tion which was entirely precipitable by brucine was designated as PS. The fraction that had originally remained in solution upon the addition of brucine and which had been precipitated by pyri-dine, was redissolved in acetic acid and the brucine solution added again. A precipitate formed. It was separated by centrifugation and the insulin material remaining in solution precipitated by the addition of pyridine. The latter precipitate was again dissolved in acetic acid, brucine added, etc. This was repeated until the addition of brucine failed to give a precipitate. This fraction which contained practically no material precipitable by the bru-cine solution was labeled PR. The pyridine precipitates from the PS series and the brucine precipitates from the PR series were. collected together into one fraction and called PU. The three fractions were purified by pyridine-acetic acid precipitations until the products no longer gave a test for brucine by the nitric acid test. In spite of this complete separation of the two fractions, PS possessed a very high degree of potency. It contained about 30 clinical units per mg., almost as great as that of PR. The latter had a potency of about 40 clinical units per mg. and within the

accuracy of animal testing it was not distinguishable in activity from PU. PS was light brown in color and required more acid for solution than either PR or PU. The latter products were almost white. The yields of the products were as follows:

PR.. 0.317 gm.
PU.. 0.636 "
PS.. 0.360 "

Hydrolysis of Insulin.

In our first hydrolyses of insulin 15 to 20 per cent sulfuric acid was used. Upon addition of sulfuric acid a precipitate began to flock out at a concentration of about 5 per cent. On heating, it first seemed to clear somewhat and then reprecipitate as if it had coagulated. It was very slow to dissolve. Although the hydrolysates gave a negative biuret after 28 hours of heating, a precipitate formed on saturation with sodium carbonate. The hydrolysis had proceeded far enough to break down the linkages responsible for the biuret test, yet the material was not broken down to such an extent that it was not precipitated by sodium carbonate.

20 per cent hydrochloric acid was found to be much superior to sulfuric acid for the hydrolysis of insulin. With the addition of hydrochloric acid to the insulin solution a precipitate formed at a concentration of about 3 per cent. As the concentration was increased the precipitate seemed to grow less and at 20 per cent the solution had cleared considerably but not entirely. Upon heating, the solution cleared to a great extent and with cooling a precipitate formed again. It was found that after 2 hours heating in a boiling water bath the insulin solution became biuret-free. In the following experiments 4 hours were allowed to insure ample time for hydrolysis.

Phosphotungstic Reaction on Hydrolyzed and Unhydrolyzed Insulin and a Possible Method of Assay.

A study was made to correlate if possible the intensities of the phosphotungstic reaction on hydrolyzed and unhydrolyzed insulin preparations with the potencies of the products. The Hunter (14) modification of the Folin-Looney technique was employed. It was hoped that such a study might reveal a chemical assay for

insulin or at least show whether or not a parallelism existed between the strength of the reactions and the potency.

To aliquots of the neutralized hydrolysate or solutions of insulin containing about 2 mg. of the substance, 1.4 cc. of 0.1 N sodium hydroxide, 0.1 cc. of 20 per cent lithium sulfate, enough water to make a volume of 3.5 cc., and then 2 cc. of 20 per cent sodium sulfite were added. After standing 5 minutes, 0.5 cc. of the phosphotungstic reagent of Folin and Trimble were added with shaking. They were then allowed to stand 10 minutes for color development, diluted to 20 cc., and compared in a colorimeter with standards containing 0.2 mg. of cystine treated in the same manner.

The percentages of cystine found are compared in Table I

TABLE I.

Phosphotungstic Reaction and Potency.

Insulin preparation.	Per cent cystine equivalents.		
	Unhydrolyzed.	Hydrolyzed.	Clinical units per mg.
U–273	5.3	7.0	15
U–440	5.9		18
PK	6.0	6.3	20
PS	9.0	9.2	30
PR	9.8	11.2	40
PU	12.2	13.2	40
CA	3.0		2
DA	2.0		1

with the potencies of the products. The values shown are averages of a number of determinations. The rabbit testing on the highly purified preparations is extremely unsatisfactory. The column indicating unitage shows, however, the general order of the potency of the products. The color reaction with the hydrolyzed material is stronger than that with the unhydrolyzed, but seems to run quite parallel to it. The study brings out distinctly an increase in the strength of the reactions with increasing potency. It is very encouraging as a possible method of assay. It is admittedly unsuited for very impure samples containing large amounts of protein impurities. So far this method has not been tried on insulin inactivated by ultra-violet light. Insulin inactivated by alkali however gives only a very weak reaction if any.

The fact that insulin inactivated by special means still responds chemically in an *in vitro* method does not necessarily rule that particular method out.   If this inactivation, however, could occur in the ordinary procedures used in dealing with insulin then it would be, of course, a serious objection.

A somewhat analagous case might be presented for the chemical method of epinephrine in which a blue color is produced with a phosphomolybdic reagent.   It is possible to destroy or at least greatly attenuate the physiological activity of epinephrine without affecting the phenolic groups responsible for the *in vitro* reaction.   Nevertheless, the method is a very valuable one.

In the same way an *in vitro* method of assay for insulin, such as the one suggested here, may be of great value in following the insulin through certain purifications and manipulations, and when free enough from associated proteins be an actual aid in the assaying of the amount of insulin present.

Direct Reaction (Labile Sulfur).

In some of the preliminary work on the phosphotungstic reaction with the sodium carbonate method, it was noticed that heating with the sodium carbonate increased the strength of the reaction.   This fact was evidently due to the labile sulfur and seemed to be worth while investigating as a means of assaying insulin.

It was demonstrated that by heating with 0.1 N sodium carbonate the sulfur is split off in such a form as to reduce the phosphotungstic reagent directly.   With insulin heated with carbonate it was unnecessary to reduce first with sulfite.   This suggests that the sulfur is either split off as a sulfhydryl group or as sulfide ion, other evidence pointing more strongly towards the latter.   With samples of cystine, heated in the same manner for 45 minutes with 0.1 N sodium carbonate, no direct reaction was obtained, demonstrating that the sulfur of cystine is comparatively stable.

The preliminary studies of this direct reaction did not give much promise for a chemical method of analysis, but nevertheless brought out a very interesting and important fact concerning the lability of the sulfur in hydrolyzed insulin preparations.   Insulin hydrolyzed with acid behaved like the original insulin towards the phosphotungstic reagent.   Only after reduction with sodium

sulfite did it respond to this test. In marked contrast, however, with the original insulin the sulfur of the hydrolysate was found to be comparatively stable. Heating with 0.1 N sodium carbonate failed to produce the direct reaction just as it did in the case of cystine.

In order to eliminate the possibility of the influence of salt present in the hydrolysate resulting from the neutralization of the acid, a few mg. of insulin were added to the sample of hydrolysate. Heating with alkali immediately produced the direct reaction, showing that if the sulfur of the hydrolysate had been labile the presence of much sodium chloride would not have prevented its demonstration.

This clearly indicates that in the insulin molecule there is a sulfur-containing moiety which can be broken off by acid hydrolysis. The sulfur of this fragment is comparatively stable toward alkali, while that in the intact molecule is labile. This observation further implies that if the active principle actually contains sulfur it cannot be a simple chemical entity adsorbed on the protein but must be a complex substance, the sulfur of which is labile. When this complex is hydrolyzed by acid, the sulfur of the sulfur-containing portion becomes stable, due to the absence of the influence of the other groups with which it had been attached. This is very similar to the behavior of the sulfur of cystine when the latter is linked to other amino acids and when it is in the free state, as shown by Brand and Sandberg.

Source of the Labile Sulfur.

If this sulfur had as its source the disulfide linkage responsible for the phosphotungstic reaction, we would expect, after treatment with alkali and elimination of the sulfur formed as hydrogen sulfide, that the phosphotungstic reaction would be decreased. On the other hand, if this reaction retained its intensity after such treatment, the sulfur split off must have been present in the molecule in some other form than the disulfide.

Insulin preparations were heated in a current of nitrogen with 0.1 N sodium carbonate for 45 minutes in a boiling water bath. Acid was then added to liberate the hydrogen sulfide which was determined by an iodometric method (15). The material left in

the test-tube after the hydrogen sulfide had been driven off was then evaporated and made up to a volume of 3 cc. containing 20 per cent hydrochloric acid.   It was next hydrolyzed under a condenser for 4 hours in a boiling water bath.   The evaporated hydrolysate was dissolved in water and the disulfide sulfur determined by the Hunter modification.

After the labile sulfur had been driven off, the disulfide content was most strikingly reduced.   Sample PU which had given a value of 13.2 per cent cystine equivalents on the hydrolyzed material, contained only the equivalent of 2.2 per cent cystine after the above treatment.   U-273, which had an original value of 7 per cent, dropped to 1.6 per cent.   The amount of hydrogen sulfide driven off was about 32 per cent of the total sulfur.   The latter was found to be about 2.8 per cent by the sodium carbonate-sodium peroxide fusion method as used by Blatherwick (16).   In this method the sulfur is determined as $BaSO_4$ nephelometrically which is not particularly satisfactory.   The value of 2.8 per cent sulfur if calculated as cystine would be equal to about 10.4 per cent cystine.   This does not account for all the phosphotungstic reaction if the latter is calculated as cystine.   As we shall show later, there is reason to believe that there is present in the material some substance other than cystine that gives the phosphotungstic reaction under conditions to which cystine reacts.

Sullivan Reaction.

As we have stated the phosphotungstic reaction cannot be considered specific for cystine, for other disulfides can give the reaction.   Sullivan (17) has recently found a specific reaction for cysteine.   At least he has so far been unable to find any other compound (and he has tested many) that gives this reaction. Cysteine, after reduction by sodium cyanide, gives the reaction.

The cystine content of the insulin hydrolysate was determined quantitatively by this method, a solution of cystine being used as a standard.   The unhydrolyzed insulin gave a negative Sullivan reaction.

One of the experiments will be given.   30.1 mg. of PU were dissolved in 1 cc. of 0.1 N HCl and 2 cc. of 30 per cent HCl added carefully.   The mixture was heated for $4\frac{1}{2}$ hours in a boiling water

bath. After evaporating to dryness, it was dissolved in 6 cc. of water.

Of this solution 2.4 cc. were introduced into a test-tube and after being neutralized were diluted to 5 cc. with enough HCl to make the solution 0.1 N, and 1 cc. of 5 per cent NaCN was added and the mixture allowed to stand 10 minutes. To this, 1 cc. of 0.5 per cent aqueous solution of the β-naphthoquinone sulfonate was added, followed by 5 cc. of a 10 per cent solution of sodium sulfite in 0.5 N NaOH. A red-brown color developed on standing 30 minutes which changed to a cherry-red color upon the addition of 1 cc. of a 2 per cent solution of sodium hyposulfite, $Na_2S_2O_4$, in 0.5 N NaOH. Five standards of cystine were run at the same time containing from 0.4 to 2.0 mg. at 0.4 mg. intervals. The standards were in 5 cc. volumes of 0.1 N hydrochloric acid. The color produced in the insulin solution was very close to that produced in the tube containing 0.8 mg. of cystine. With the latter set at 10 the unknown read 9, giving a cystine value of 0.888 mg. in the unknown, which represents a cystine content of 7.37 per cent.

At the same time a solution of insulin was run which had been treated with 0.1 N sodium carbonate and then hydrolyzed with hydrochloric acid. Although containing the same equivalent amount of insulin the color production was almost nil. It was most striking, this difference in color of the two tubes; one was a bright cherry-red while the other was only a very pale orange. There could be no doubt that the splitting out of the labile sulfur had destroyed the compound which gave the Sullivan reaction. The source of this labile sulfur must have been this compound which, as far as we know now, is cystine, or a substance having the SH and NH_2 groups in close proximity, with the SH groups in the oxidized form; in other words, the disulfide linkage.

The phosphotungstic reaction was also run on an aliquot of the hydrolysate of the 30.1 mg. sample. The average value obtained was equivalent to 13.2 per cent cystine.

The values for cystine equivalents obtained by the two methods do not agree, the more specific reaction indicating a much lower value. There is evidently present a compound which does not give the specific reaction for cystine but which gives the phosphotungstic reaction after reduction with sulfite. This may be either

a disulfide compound different from cystine or, possibly, a dipeptide of cystine still unhydrolyzed. A compound such as the latter would not give the Sullivan reaction but would still respond to the phosphotungstic reaction.

It was conceivable that the boiling of cystine with 20 per cent HCl had so affected the molecule that although still giving the disulfide reaction, it would fail to give the more specific test. However, 10 mg. of cystine boiled for 4 hours with 20 per cent HCl gave the theoretical value with both of the methods.

<div align="center">CONCLUSIONS.</div>

If the active principle itself actually contains sulfur, as we have good reason to hold, then we believe that the evidence presented in this study indicates that this sulfur is present as the disulfide linkage and that insulin is most likely a derivative of cystine.

By means of the phosphotungstic reaction we found that as the insulin became more purified the cystine content increased. The proportionality was so striking that it has given us hope that this might prove to be a suitable means of assay for purified preparations.

When the sulfur was split out, the disulfide linkage was destroyed and the test for cystine greatly reduced in intensity, indicating this as the source of the labile sulfur.

Since it has been shown that insulin itself does not give the specific Sullivan reaction for cystine, and since we have demonstrated the presence of cystine in the hydrolyzed insulin preparations, it must be concluded that the cystine is present as some derivative. Further, the sulfur of the hydrolysate is comparatively stable like free cystine, whereas the sulfur of the original unhydrolyzed insulin is very labile. This change of lability upon hydrolysis is identical with what would be expected of an amino acid derivative of cystine. Glutathione, for instance, has a sulfur lability of the order of insulin. Upon acid hydrolysis free cystine would be formed and the sulfur of the hydrolysate would therefore be stable.

The behavior of the sulfur in insulin is quite parallel to the behavior of the sulfur in amino acid derivatives of cystine and suggests that the cystine in insulin is linked to the rest of the molecule by

a peptide linkage. In this connection the high arginine, histidine, and tyrosine contents of purified preparations might be recalled.

In Abel's analyses the calculated empirical formula of $C_{45}H_{75}O_{17}N_{11}S$ is based upon the presence of 1 sulfur atom in the molecule. From our work on the presence of the disulfide linkage the minimum value would have to be twice that given, or $C_{90}H_{150}O_{34}N_{22}S_2$.

The writer wishes to express his appreciation to Professor J. R. Murlin for his interest and helpful suggestions during the course of this investigation.

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THE CHEMICAL STUDIES OF THE OVARY.

XIII. THE WATER-SOLUBLE EXTRACTIVES OF OVARIAN RESIDUE.

By FREDERICK W. HEYL AND MERRILL C. HART.

(From the Research Laboratories, The Upjohn Company, Kalamazoo.)

Considerable confusion exists in ovarian therapy as to the correct form of ovarian extract for clinical use. Various forms of ovarian extract are commercially available and these vary in nature from material representing the whole extract to preparations containing only the water-soluble material of various parts of the gland. All of these products are claimed to be physiologically active. This condition in the commercial field reflects the same condition which is found in the scientific literature. Considerable evidence has been accumulated to prove that the physiologically active material of the ovary is lipoidal in nature. The activity of the water-soluble fractions could be explained on the assumption that this active lipoidal constituent of the gland was also water-soluble.

During the course of some work in this laboratory on the lipoids of corpus luteum and ovarian residue, the opportunity was given for the comparison of the chemistry of the water-soluble fractions of these two materials. This information is important due to the increasing use of these fractions hypodermically. We are reporting here the results from the study of the water-soluble fraction of ovarian residue. The study of this same fraction from corpus luteum will be reported on later.

EXPERIMENTAL.

4.53 kilos of finely ground ovarian residue were extracted with 100 liters of ether. The ether was removed and the dried gland percolated with absolute alcohol (A). The residue from the alcoholic extract was macerated with 30 liters of water and heated

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to 80°C. for 30 minutes.   After cooling, the supernatant extract
was removed, and the extraction process repeated twice on the
residue.   These extracts were coagulated completely by acetic
acid, filtered, and the filtrate and washings from the coagulum
concentrated to a volume of 4 liters.   This solution (B) was pre-
served with a small amount of normal lead acetate solution.

Examination of the Alcohol Extract (A).—The alcoholic solution
on concentration yielded three fractions of insoluble material.
The water extract of this insoluble material consisted of isoleucine
and sodium chloride.[1]   The filtrate and washings from the insolu-
ble material were concentrated and poured into ether.   An insol-
uble precipitate (C) was formed.   This was filtered off and the
ether filtrate extracted with water.   The precipitate (C) was
emulsified with water and the emulsion extracted with ether and
chloroform.   This water extract of the precipitate (C) was joined
with the water extract of the ether-soluble material for further
study (D).

Examination of the Water Extracts (D).—This clear water solu-
tion was concentrated to a volume of 1 liter.   Analysis of an
aliquot of this solution gave the following results.

	gm.
Creatinine[2] (after hydrolysis)	2.63
Urea[3]	2.99
Ammonia[3]	1.53
Total nitrogen	6.32
Basic " (phosphotungstic acid-precipitable)	2.55
Non-basic nitrogen	2.51

Of the non-basic nitrogen, urea and creatinine nitrogen account
for all but 0.10 gm. of this fraction.

The remainder of the solution (900 cc.) was clarified with normal
lead acetate solution.   The excess lead was removed as the sul-
fide and the basic nitrogen precipitated as the phosphotungstate
(E) in the usual manner.

Examination of the Phosphotungstic Acid Precipitate (E).—
Aliquot determinations on the filtrate from the barium phos-

[1] Heyl, F. W., and Fullerton, J. B., *J. Am. Pharm. Assn.*, 1926, xv, 549.

[2] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

[3] Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, 1914, lxii, 1558.

photungstate, after decomposition of the phosphotungstate, gave
the following results.

	gm.
Total solids	20.3
Nitrogen	2.5
Ash	4.2
Creatinine[2] (after hydrolysis)	1.7

The solution of the phosphotungstic acid-precipitable material
was made acid with nitric acid and the purine silver fraction pre-
cipitated with silver nitrate solution. The histidine and arginine
fractions were separated, in the usual manner, with silver nitrate
solutions and barium hydroxide. The lysine fraction was ob-
tained from the filtrate from the arginine fraction, after clarifica-
tion from reagents, by precipitation with phosphotungstic acid.

Examination of the Purine Silver Fraction.—This fraction
weighed 2.7 gm. This was digested with warm 10 per cent
ammonia water and the insoluble residue suspended in water,
the excess ammonia removed, and decomposed with hydrogen
sulfide. The filtrate from the silver sulfide was evaporated to
dryness, dissolved in dilute hydrochloric acid, and decomposed at
the boiling point with ammonia in excess. On cooling, white
crystalline material separated, which was washed with 2 per cent
ammonium hydroxide solution. This weighed 0.344 gm. This
was again dissolved in dilute acid and precipitated again at the
boiling point with ammonia. This was filtered off, washed, and
dried. Weight, 0.163 gm. This did not give the nitric acid test
for guanine.

This material was converted into the picrate and crystallized
six times from water. 0.117 gm. of yellow needles was obtained.
These melted at 256–258°C. Analysis for picric acid by the nitron
method gave the following results.

Analysis.
0.1096 gm. substance: nitron picrate 0.1572.
Calculated for $C_5H_5N_5 \cdot C_6H_3O_7N_3$. Picric acid 62.9.
Found. " " 60.7.

This material is impure adenine picrate. Hypoxanthine and
xanthine were not present in this purine silver fraction.

Examination of the Histidine and Arginine Fractions.—These

fractions were examined separately. The total histidine, determined colorimetrically,[4] amounted to 22.9 mg. Mercuric sulfate precipitation, picrolonic acid, and gold salt formation failed to yield material of interest.

Examination of the Lysine Fraction.—The phosphotungstate representing this fraction was washed and decomposed in the usual manner. The filtrate from the barium phosphotungstate was clarified from reagents and concentrated to a syrup. This syrup was dissolved in absolute alcohol containing 2 per cent dry hydrogen chloride and concentrated to dryness *in vacuo*. This process of dissolving in alcoholic hydrochloric acid and evaporating to dryness was repeated three times. The alcoholic solution of the chlorides was precipitated with a saturated alcoholic solution of mercuric chloride. The crystalline mercury salt was centrifuged off and washed with alcohol. The filtrate from the mercury salt was made alkaline with barium hydroxide and the lysine fraction (F) precipitated.

The crystalline mercury salt was crystallized from hot water. The crystalline material was suspended in water and decomposed with hydrogen sulfide. The filtrate from the silver sulfide yielded on evaporation 1.287 gm. of long silky needles. 0.50 gm. of this was converted into 0.756 gm. of yellow needles of the gold salt. These on crystallization from dilute hydrochloric acid gave 0.181 gm. of beautifully crystalline needles. These crystals were dried to constant weight and analyzed.

Analysis.
0.1756 gm. substance: Au 0.0778.
Calculated for $C_5H_{14}NOCl \cdot AuCl_3$. Au 44.5.
Found.   " 44.3.

The water filtrate from the crystallization of the choline mercury salt, after removal of the mercury, yielded 2.385 gm. of long silky needles of the chloride. From this was prepared 0.935 gm. of the gold salt. This on crystallization gave 0.204 gm. of yellow needles of the purified choline gold salt. These also analyzed for choline gold chloride.

Analysis.
0.1949 gm. substance: Au 0.0862.
Calculated for $C_5H_{14}NOCl \cdot AuCl_3$. Au 44.5.
Found.   " 44.2.

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[4] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, xxxix, 497.

Examination of the Lysine Fraction (F).—The mercury was removed from this fraction and the solution precipitated by phosphotungstic acid. The phosphotungstic acid-precipitable material was evaporated to dryness several times with absolute alcohol and finally dissolved in 5 cc. of alcohol and treated with an alcoholic solution of picric acid. Small clumps of crystalline material separated. These were crystallized thrice from 3 cc. of alcohol. 0.0379 gm. of balls of microcrystalline material was obtained. These were analyzed for picric acid.

Analysis.
 0.0379 gm. substance: picric acid 0.0251.
 Calculated for $C_5H_{11}NO_2 \cdot C_6H_2(NO_2)_3OH$. Picric acid 66.2.
                                    Found. "   "   66.3.

This substance analyzes for a picrate of δ-aminovalerianic acid.

Examination of the Filtrate from the Phosphotungstate (E).—This was clarified from reagents in the usual manner. There were 2.51 gm. of nitrogen present in this solution. This nitrogen is made up largely of creatine and urea, as we have shown.

The total solids present in this solution amounted to 55.4 gm. This fraction was split into an alcohol-soluble and an alcohol-insoluble fraction with absolute alcohol. This alcohol-insoluble material weighed 5.47 gm., of which 4.18 gm. were inorganic, consisting mainly of NaCl. Glucose was not present in this material. A lead subacetate precipitation of this material after a preliminary clarification with lead acetate gave no indications of the presence of inosite.

The alcohol was removed from the alcohol-soluble part of this material and the residue dissolved in water and neutralized with a solution of barium hydroxide. The ether extract of this neutral solution removed only a trace of oily material. The solution was then made strongly acid with phosphoric acid and extracted with ether. The residue from the ether extract was boiled for 30 minutes with an excess of freshly precipitated lead carbonate, filtered, and the filtrate decomposed with hydrogen sulfide. The filtrate from the lead sulfide was concentrated and boiled for 30 minutes with an excess of freshly precipitated zinc carbonate. The solution was filtered, concentrated to incipient crystallization, and alcohol added. A white crystalline salt separated. This was filtered off, washed with alcohol, and dried.

Weight, 8.78 gm.   This was purified by a second crystallization from dilute alcohol.   It was dried to constant weight *in vacuo* and analyzed.

Analysis.
    0.4938 gm. substance: ZnO 0.1438.
    0.7383 "   "   : H_2O at 105°C., 0.0990.
    Calculated for $(C_3H_5O_3)_2Zn \cdot 2H_2O$.   ZnO 29.0, H_2O 12.9.
                                    Found.   " 29.1,   " 13.4.
    4.1757 gm. of this material made up to 50 cc. were optically active. $[\alpha]_D^{20} =$ $-5.4.°$

This material is the zinc salt of δ-lactic acid.

Examination of Water Extract (B).—This extract solidified to a solid gel upon standing.   It was diluted to 40 liters.   Nothing could be directly crystallized from this extract after it had been treated with an excess of tannic acid, baryta, sulfuric acid, and lead oxide as in the method of Kutscher.[5]   The solution was concentrated, made acid with sulfuric acid, and precipitated with phosphotungstic acid.   Systematic examination of this phosphotungstate acid fraction (H) showed the presence of creatinine, guanine, adenine, histidine, arginine, carnosine, and lysine.[1]

Examination of the Filtrate from the Phosphotungstate (H).— This was freed from reagents in the usual manner and concentrated to 500 cc.   Analysis on an aliquot of this solution showed the presence of 6.52 gm. of total nitrogen, 0.55 gm. of ammonia nitrogen, and 4.51 gm. of amino nitrogen.

A portion of the solution containing 0.326 gm. of total nitrogen was hydrolyzed with hydrochloric acid and examined by the Van Slyke process with the following results.

	per cent
Amide nitrogen	6.23
Humin "	5.5
Total base nitrogen	14.5
Non-basic "	72.8
" amino nitrogen	62.2
" non-amino nitrogen	10.6

Hydrolysis does not increase ammonia nitrogen but produces considerable humin.   Some base is liberated, however, as 14.5 per

⁵ Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin, 1910, ii, 1044.

cent of nitrogen is rendered precipitable with phosphotungstic acid.

The following colorimetric determinations were made on aliquots of the solution.

Tryptophane[6]..Negative.
Tyrosine[7]... 0.09 gm.
Cystine[6]... 0.81 "

The remainder of the solution was hydrolyzed by boiling for 18 hours with 20 per cent sulfuric acid, filtered free from humus (12.13 gm.), and precipitated with phosphotungstic acid in the usual manner. This phosphotungstate (I) was centrifuged off, washed, and decomposed in dilute acetone solution.

Examination of the Phosphotungstate (I).—This solution was freed from reagents, concentrated to 150 cc., made acid with nitric acid, and separated into the usual fractions with silver nitrate and barium hydroxide. The lysine fraction was isolated by precipitation with phosphotungstic acid of the filtrate from the arginine fraction.

Examination of the Purine Fraction.—By decomposition of the soluble chlorides obtained by decomposition of the silver compound a guanine fraction was obtained. This formed a light brown, semicrystalline material that gave a positive test with nitric acid and sodium hydroxide. The diazo reaction was strongly positive. By treatment of a water solution of the chloride of this fraction with sodium picrate, 0.0336 gm. of the picrate was formed. This material is probably guanine.

The filtrate from the guanine fraction was boiled to remove excess ammonia, made slightly acid with hydrochloric acid, and to the hot solution, a solution of sodium picrate was added. Long crystalline needles formed. These were filtered off and on recrystallization from water gave 0.0147 gm. of adenine picrate. These crystals decomposed at 278°C.

Hypoxanthine and xanthine were absent in the filtrate from the adenine picrate.

Examination of the Histidine and Arginine Fractions.—This fraction contained 0.0128 gm. of nitrogen. Systematic study gave no indication of the presence of histidine or arginine.

[6] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.
[7] May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, liv, 213.

Examination of the Lysine Fraction.—This fraction was decomposed in the usual manner. The chlorides were all soluble in absolute alcohol. No picrate could be formed. The picric acid was removed, the solution evaporated to dryness, dissolved in a little alcohol, and precipitated with 4 cc. of a saturated alcoholic solution of mercuric chloride. The heavy voluminous precipitate was filtered off, washed with a little alcohol, and crystallized from water. The crystalline mercury salt and water filtrate were separately decomposed by hydrogen sulfide, and the filtrates from the mercury sulfide evaporated. Crystalline residues of 0.375 and 0.614 gm. respectively were obtained. These were converted into the gold salts and crystallized from dilute hydrochloric acid. Yellow crystals were obtained that weighed 0.384 and 1.231 gm. These decomposed sharply at 261°C. On heating, the odor of trimethylamine was obtained. The first fraction was analyzed.

Analysis.
    0.1723 gm. substance: Au 0.0765.
    Calculated for $C_5H_{14}NOCl \cdot AuCl_3$. Au 44.5.
                          Found.   " 44.4.

This material is choline gold salt.

Examination of the Filtrate from the Phosphotungstic Acid Precipitate (H).—This filtrate was freed from reagents and extracted with butyl alcohol. Both the butyl alcoholic extract and the aqueous solution were esterified.[8] These were joined and distilled fractionally at 1.2 mm. pressure. Three fractions and a residue were obtained. The entire yield was about 1.5 gm. and most of this was in the lowest fraction and in the liquid air trap. These were joined, saponified by boiling with water, evaporated to dryness, and extracted with absolute alcohol. Fractions II, III, and the residue were treated in a similar manner.

Fraction I.—Boiling point up to 54°C.
Analysis.
    0.00768 gm. substance: cc. N at 26°C. and 7.40 mm. pressure, 2.40.
    Found. NH_2-N 16.8.
Fraction II.—Boiling point 54–110°C.
Analysis.
    0.01216 gm. substance: cc. 0.02 N NH_3 6.54.
    0.01216 "   "   " N at 25°C. and 746 mm. pressure, 3.43.

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[8] Levene, P. A., and Van Slyke, D. D., *J. Biol. Chem.*, 1909, vi, 419.

Calculated for C₂H₆O₂N.   N 18.67.
"   " C₃H₇O₂N.   " 15.73.
Found. Total N 15.07. NH₂-N 15.36.

The analyses of these first two fractions indicate a mixture of glycocoll and alanine.
The alcohol-insoluble part of the residue analyzed as follows:

Analysis.
0.0140 gm. substance: cc. N at 27°C. and 750 mm. pressure, 3.26.
Calculated for C₃H₇O₂N. N 13.34.
"   " C₄H₇NO₄. " 10.53.
Found. " 12.60.

This fraction consists of a mixture of serine and aspartic acids.
The alcohol-soluble material, on analysis, suggests the presence of proline.

Analysis.
0.02628 gm. substance: cc. 0.02 N NH₃ 9.52.
0.01314 "   "   : " N at 26°C. and 744 mm. pressure, 0.20.
Calculated for C₅H₉O₂N. N 12.17.
Found. " 10.15. NH₂-N 0.90.

SUMMARY.

Systematic examination of the water-soluble fraction of the alcoholic extract of ovarian residue showed the presence of isoleucine,[1] creatinine (after hydrolysis), urea, ammonia, choline, adenine, histidine, δ-aminovalerianic acid, and δ-lactic acid.

The water extract of the alcohol-extracted ovarian residue gave a phosphotungstic acid fraction that contained creatinine, guanine, adenine, histidine, arginine, carnosine, and lysine.[1] The filtrate from the phosphotungstic acid precipitate contained ammonia, tyrosine, and cystine. Hydrolysis of this fraction gave more basic nitrogen. This basic nitrogen consisted largely of choline with small amounts of guanine and adenine. The filtrate from the phosphotungstic acid precipitate gave indications of the presence of the amino acids glycocoll, alanine, serine, aspartic acid, and proline.

THE PRODUCTION OF GLUCONIC ACID BY THE PENI-CILLIUM LUTEUM-PURPUROGENUM GROUP. I.

By ORVILLE E. MAY, HORACE T. HERRICK, CHARLES THOM, AND MARGARET B. CHURCH.

(From the Color Laboratory and Microbiological Laboratory of the Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.)

Many investigators have reported the development of an acid reaction in sugar solutions on which fungi have been allowed to grow. When such solutions have been analyzed the acidity has been usually found to be due to varying amounts of oxalic and citric acids, and a study of the conditions under which these acids are formed has been the object of numerous investigations (1–10).

More recently gluconic acid has been isolated from sugar solutions on which *Aspergillus niger* has been cultured. Whether *Aspergillus niger* will produce citric or gluconic acids from sugar solutions seems to depend somewhat on the particular strain of the organism employed, but more on the conditions of the experiment (11–15). Very recently Bernhauer (16) carried out a systematic series of experiments with the object of ascertaining the exact conditions governing the formation of gluconic, citric, and oxalic acids by *Aspergillus niger*. By controlling the pH of the culture medium and the temperature and by a proper choice of inorganic nutrient salts he found it possible to obtain good yields of either gluconic or citric acids at will.

Heretofore research on the oxidation of sugar solutions by fungi has been confined mainly to a few strains of *Aspergillus niger* and species of Citromyces with very few scattered and rather casual references to the *Aspergillus glaucus* group, *Penicillium glaucum*, and *Penicillium arenarium*. In view of the many acids that may possibly result from the oxidation of glucose it was thought desirable to investigate this field further, especially the growth of fungi other than *Aspergillus niger* on glucose solutions.

Advantage was taken of the excellent collection of various fungi maintained by Drs. Thom and Church in the Microbiological Laboratory of the Bureau of Chemistry where most of the work reported in this paper was carried out.

The glucose solutions employed were of varying concentrations but consisted for the most part of 10, 20, and 30 parts of commercial glucose per 100 parts of solution. This glucose contained 91.5 per cent dextrose and 8.0 per cent moisture. Inorganic salts were added in the following amounts.

<div style="text-align:right">Gm. per liter of
glucose solution.</div>

$MgSO_4 \cdot 7H_2O$...	0.5
K_2HPO_4..	0.1
KCl..	0.1
$NaNO_3$..	1.0

The cultures were made in 200 cc. Erlenmeyer flasks each containing 75 cc. of glucose solution. They were sterilized with steam at 15 pounds pressure for 15 minutes. At this temperature solutions of the higher concentrations of glucose assumed a light yellow tinge while the lower concentrations (10 and 20 parts) were not affected. Each fungus studied was cultured for 3 weeks on at least three concentrations of glucose (10, 20, and 30 parts) and in some cases on solutions of eight different concentrations up to saturated solutions. It was found, as would be expected, that the growth of the fungi was greatly inhibited in concentrations of over 40 parts of glucose, although in the majority of cases the organism grew to some extent.

In all, 172 different strains of fungi were studied including the genera *Aspergillus*, *Penicillium*, *Monilia*, and *Mucor*. Production of acid in appreciable quantities was found to be limited to a few strains of *Aspergilli* and *Penicillia*. Under the conditions of our experiments certain strains of *Aspergilli*, namely *niger*, *glaucus*, *tamarii*, and *wentii*, produced citric and oxalic acids in varying amounts. All of the solutions on which the *Monilia* were cultured became alkaline after 1 week. All of the *Mucores* produced slightly acid solutions after 2 weeks. No attempt was made to separate and identify the small amounts of acid formed in these solutions but fumaric acid was probably present. Appreciable quantities of this acid as well as oxalic acid have been isolated from glucose solutions on which *Mucor stolonifer* has been

cultured (15, 17, 18). Some of the fungi, notably certain strains of the *Aspergillus flavus* group, developed pleasant fruity odors of considerable intensity suggestive of pineapple. Other solutions developed a beautiful fluorescence and many strains of the *Penicillium luteum-purpurogenum* and *Aspergillus glaucus* groups gave positive results when tested with $FeCl_3$ solution.

The only fungi other than the *Aspergilli* producing acid in large amounts were certain strains of *Penicillia*. Among these may be mentioned *Penicillium luteum-purpurogenum, divaricatum*, and *citrinum* groups. Gluconic and citric acids were isolated from solutions on which *Penicillium citrinum* and *Penicillium divaricatum* were cultured. A small but appreciable quantity of a third acid, soluble in water and insoluble in alcohol and melting above 200°C., was also isolated from these solutions. Crystallographic measurements indicated that this acid was not one of the common oxidation products of sugar and as yet it has not been positively identified.

The solutions on which strains of *Penicillium luteum-purpurogenum* were cultured all became strongly acid after 7 days. Analysis of these solutions gave no indication of the presence of either citric or oxalic acids. Gluconic acid was recovered from these solutions, as the calcium salt, in amounts sufficient to account for the entire titratable acidity, taking into consideration the unavoidable losses due to its solubility. The following procedure was used in separating and identifying the salts of gluconic acid.

Calcium Gluconate.—The mycelium was separated from the culture medium by filtration and was thoroughly washed with warm water after which it was pressed out on the filter paper to remove any small amounts of acid still absorbed in the tissue. The washings were then combined with the filtrate.

A portion of this solution was tested for the presence of citric and oxalic acids with negative results. Tests were also made for tartaric and malic acids and no indication of their presence was ever observed. $CaCO_3$ was then added to the remainder of the culture liquor and after the evolution of CO_2 had ceased the solution was filtered. The filtrate was heated to boiling to remove any $Ca(HCO_3)_2$ and again filtered. Since the filtrate was colored a rather deep yellow due to the boiling charcoal was added, the solution warmed, and after filtration the color had completely dis-

appeared. An equal volume of 95 per cent ethanol was then added to the filtered solution slowly and with stirring. On standing for 18 hours the calcium gluconate came down as a rather voluminous precipitate. After filtration the salt was dissolved in 6 parts of hot water and 2 parts of 95 per cent ethanol were added. After 2 hours the calcium gluconate began to settle out on the sides of the container as a crusty mass of fine crystals and after 24 hours the crystallization was complete. The precipitate was washed with 50 per cent ethanol and air dried on a Buchner funnel after which it was placed in an oven at 40°C. for 10 days. A sample of salt dried in this manner lost no weight on heating at 105°C., indicating that calcium gluconate contains no water of crystallization. CaO was determined by ignition.

Ca in $Ca(C_{12}H_{11}O_7)_2$.   Calculated.   9.30 per cent.
Found.   9.21,   9.25   "   "

The specific rotation was determined by dissolving 3 gm. of the salt in H_2O to make exactly 100 cc. of solution at 20°C. and making the determination in a saccharimeter at 20°C., the specific rotation being calculated from the usual conversion equation. The rotation was determined both within 1 hour after the solution had been made up and after standing for 24 hours. Identical values were obtained in both cases. $[\alpha]_D^{20} = + 9.8°$. This value is in fair agreement with the later determinations reported in the literature. Nef (19) reports $[\alpha]_D^{20} = + 10.5°$, van Ekenstein and coworkers (20) give a value of $+9.9°$, Falck and Kapur (14), $+9.7°$, and Butkewitsch (13), $+6.3°$. Butkewitsch (15) reports $[\alpha]_D^{22} = + 7.8°$.

Calcium gluconate was also prepared by concentrating the neutralized culture liquors to a sirupy consistency and placing the sirup in a cool place whereupon the salt separated from the solution. After filtration it was vigorously stirred in 50 per cent ethanol and the mixture filtered. This operation was repeated using fresh ethanol and after drying the final product was of good quality but not quite as white in color as the salt prepared by precipitation with ethanol.

. *Barium Gluconate.*—The barium salt was prepared by neutralizing the culture liquor with $BaCO_3$ and precipitating the gluconate with 4 volumes of 95 per cent ethanol. This salt forms beautiful

rhombohedral crystals when crystallized from ethanol-water mixtures. It apparently contains 1 molecule of water of crystallization. Samples dried in a vacuum desiccator over H_2SO_4 for 2 days and then 1 day at 65° in the oven gave the following results upon analysis.

Ba in $Ba(C_6H_{11}O_7)_2 \cdot H_2O$.   Calculated.   25.18 per cent.
Found.   25.22,   25.30   "   "

Another sample of the barium gluconate was dried at 105°C. and the Ba determined as $BaSO_4$.

Ba in $Ba(C_6H_{11}O_7)_2$.   Calculated.   26.05 per cent.
Found.   26.10,   26.22   "   "

The specific rotation was determined by the same procedure used in the case of the calcium salt. $[\alpha]_D^{20} = + 9.0°$. No values of $[\alpha]$ for barium gluconate are reported in the literature.

A number of the alkali, alkaline earth, and metallic salts of gluconic acid as well as some organic derivatives are being prepared and purified and a report of their physical constants will be made in a forthcoming publication.

The amount of gluconic acid produced varied greatly with the strains within this group, ranging from a 20 per cent yield (calculated from the glucose) to as high as 60 per cent after 14 days. The strains producing low yields of the acid also gave rise to extremely mucilaginous solutions.

One strain of the group being considered (*Penicillium purpurogenum* var. *rubrisclerotium* Thom, No. 2670) has been selected for further study as to the optimum conditions for a high production of gluconic acid. Preliminary experiments indicate that a yield approaching 80 per cent on 20 and 25 per cent glucose solutions may probably be expected in from 12 to 14 days depending on the temperature and the ratio of the surface area of mycelium to the volume of the solution. The variables affecting the production of the gluconic acid by this fungus are being worked out and will be the subject of a later paper.

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ANIMAL CALORIMETRY.

THIRTY-SIXTH PAPER.

THE EFFECT OF INSULIN ON THE METABOLISM OF DOGS UNDER AMYTAL ANESTHESIA.*

BY WILLIAM H. CHAMBERS,† HARRY J. DEUEL, JR., AND ADOLPH T. MILHORAT. .

WITH THE TECHNICAL ASSISTANCE OF JAMES EVENDEN.

(*From the Department of Physiology, Cornell University Medical College, New York City.*)

That insulin injected into the normal animal causes an increased combustion of carbohydrate is a general conclusion drawn from the large amount of experimental work recently summarized in the review of Grevenstuk and Laqueur (1) and in the monograph of Macleod (2). There is sufficient variation however in the results of the individual experiments to leave the question unsettled. Several reasons may account for the differences observed. A diversity of experimental animals has been used; *i.e.*, mice, rats, guinea pigs, rabbits, cats, and dogs. In some cases the methods employed have been inadequate for a complete and accurate picture of respiratory reactions. The nutritive condition of the animal may be an important factor according to the recent data of Chaikoff and Macleod (3). They report that in normal well fed rabbits with a respiratory quotient of unity insulin produced no increase in carbohydrate metabolism, whereas in fasted ones it caused a rise in respiratory quotient and also in oxygen consumption. Previously Hawley and Murlin (4) had found in fasted rabbits definite evidence of an increase in respiratory quotient which occurred during the 2nd hour after insulin but a diminution in the oxygen intake.

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* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, 1925, xxii, 424.

† Fellow in Medicine, National Research Council, 1924–26.

From the standpoint of heat production the majority of the experimental work is of little value due to the hypersensitivity and convulsions of the animals. This phase of the problem has been studied by Krogh and Brandt-Rehberg (5) in rabbits using curare and recently in decerebrate and decapitate cats with the same drug by Taylor and Olmsted (6). Both investigations have shown that when muscular action is prohibited there is no definite change in heat production.

In view of the opinion held by some authors that the administration of insulin to the normal animal is always followed by an increased combustion of carbohydrate, the differences in the results of the various workers constituted, in our opinion, sufficient reason for the further investigation of the problem. We are reporting in this paper experiments on the effect of insulin on the respiratory exchange, the blood sugar, and the alkali reserve of fasting dogs. Amytal anesthesia was used as it effectively prevents hypoglycemic movements. The control experiments which have been published in a separate paper (Deuel, Chambers, and Milhorat (7)) show that when sufficient amytal was given to prevent shivering, the metabolism was maintained at a fairly constant level slightly below that of the basal.

Methods.

The metabolism experiments were conducted in the respiration calorimeter of this department on four mongrel female dogs. The animals were fasted a minimum of 48 hours to insure a basal respiratory quotient of about 0.75. The amytal was injected intraperitoneally as previously described (7) and the dog placed in the calorimeter for the preliminary control period. The metabolism under amytal was followed for 2 hourly periods, and the experiment interrupted to inject the insulin (5 to 7 units per kilo of body weight) subcutaneously. About 30 minutes elapsed after the insulin injection before the observations could be started again. They were then continued for 4 hourly periods. Blood samples for sugar and alkali reserve determinations were taken from the jugular vein before giving the amytal and after the completion of the respiration experiment.

For a more accurate study of the blood and urinary nitrogen changes after insulin the experiments were repeated outside the

calorimeter, at which time the urine was collected by catheter and blood samples of 10 cc. were drawn from the antibrachial, cephalic, or small saphenous veins at hourly intervals.   The Shaffer-Hartmann method was used for blood sugar, the usual macro-Kjeldahl procedure for urinary nitrogen, and the Van Slyke technique for the alkali reserve determinations.   The blood was equilibrated three times at room temperature with alveolar air. On two of the dogs (Nos. 30 and 34) respiratory and blood and urine experiments were performed.

Respiration Experiments.

From the standpoint of the respiratory quotient we can divide our ten experiments into three groups: four experiments in which there was an increased respiratory quotient during the 2nd hour after insulin, four in which there was no significant change in quotient, and two which showed a higher quotient during the 4 hours after the insulin.

The first group is represented by the experiment on Dog 30 given in Table I.   The average respiratory quotient of the 3 preliminary hours was 0.74.   Following the insulin injection the 1st, 3rd, and 4th hours are essentially the same, 0.78, 0.75, 0.76, while the 2nd hour is distinctly higher, 0.89.   The average calculated heat production for the preliminary period was 12.86 calories. The 1st hour after insulin there was a rise to 13.67 calories, which increased to 18.60 calories the 4th hour, almost 50 per cent above the amytal control period.

A second experiment from the first group is shown in Table II. The distinct rise in respiratory quotient during the 2nd hour after insulin is more strikingly illustrated in this case.   This experiment is different from that given in Table I in that there was no increase in heat production after the insulin injection.

The reaction in the second group, composed of those experiments in which the respiratory quotient was practically unchanged by the injection of insulin, is shown in Tables III and IV.   The variation in heat production is similar to that found in the first group (Tables I and II).   With Dog 33 (Table III) there was a distinct rise in total calories after insulin, whereas in the case of Dog 30 (Table IV) there was only a slight increase in heat production during the 3rd and 4th hours.

Effect of Insulin on Metabolism of Dog 30 under Amytal Anesthesia.
Apr. 23, 1925.   Body weight, 8.08 kilos.

Hourly period ending.	O₂	R.Q.	Average urinary N.	Total calories.		Blood sugar.	Blood CO₂.	Remarks.
				Calculated.	Found.			
	gm.		*gm. per hr.*			*per cent*	*vol. per cent*	
						0.068	50.3	Blood sample at 8.55
11.38 a.m.	4.06	0.70	0.120	12.94*	9.34			a.m. 78 mg. amytal
12.38 p.m.	3.89	0.78		12.94*	12.31			per kilo at 9.39 a.m.
1.38 "	3.81	0.75		12.42	12.64			
Averages .	3.92	0.74		12.86	11.43			
								6 units insulin per kilo
3.28 p.m.	4.15	0.78	0.120	13.67	16.35			at 2.05 p.m.
4.28 "	4.19	0.89		14.19	14.52			
5.28 "	5.50	0.75		18.01	16.88			
6.28 "	5.66	0.76		18.60	18.28	0.042	46.7	Blood sample at 6.38
Averages .				16.12	16.51			p.m.

* Average of two hourly periods.

Effect of Insulin on Metabolism of Dog 28 under Amytal Anesthesia.
Mar. 25, 1925.   Body weight, 16 kilos.

Hourly period ending.	O₂	R.Q.	Average urinary N.	Total calories.		Body temperature.	Blood sugar.	Blood CO₂.	Remarks.
				Calculated.	Found.				
	gm.		*gm. per hr.*			*°C.*	*per cent*	*vol. per cent*	
						37.32*	0.092	51.7	Blood sample at
11.40 a.m.	5.81	0.71	0.102	18.94	19.47	36.93			9.33 a.m. 65
12.40 p.m.	6.35	0.73		20.75	18.66	36.53			mg. amytal
Averages .	6.08	0.72		19.85	19.07				per kilo at 9.37 a.m.
						36.42*			5 units insulin
2.10 p.m.	5.64	0.72	0.102	18.38	18.21	35.99			per kilo at
3.10 "	5.04	0.86		16.99	17.04	35.55			12.48 p.m.
4.10 "	5.76	0.73		18.83	19.75	35.32			
5.10 "	5.42	0.72		17.66	19.01	35.09	0.006	50.9	Blood sample at
Averages .				17.96	18.50				5.30 p.m.

* Body temperature at beginning of hour; other values at end of hour.

TABLE III.

Effect of Insulin on Metabolism of Dog 33 under Amytal Anesthesia.
Apr. 24, 1925.   Body weight, 15.50 kilos.

Hourly period ending.	O_2	R.Q.	Average urinary N.	Total calories.		Blood sugar.	Blood CO_2.	Remarks.
				Calculated.	Found.			
	gm.		*gm. per hr.*			*per cent*	*vol. per cent*	
						0.088	57.7	Blood sample at 9.25
11.38 a.m.	7.02	0.77	0.152	23.11	21.97			a.m. 65 mg. amytal
12.38 p.m.	7.22	0.79		23.94	23.72		.	per kilo at 9.42 a.m.
Averages .	7.12	0.78		23.53	22.85			
								5 units insulin per kilo
2.23 p.m.	8.31	0.78	0.152	27.49	26.70			at 1.05 p.m.
3.23 "	8.91	0.78		29.53	30.58			
4.23 "	9.11	0.75		29.95	31.64			
5.23 "	9.80	0.69		31.98	31.95	0.031	55.0	Blood sample at 5.35
Averages .		.		29.74	30.22			p.m.

TABLE IV.

Effect of Insulin on Metabolism of Dog 30 under Amytal Anesthesia.
Apr. 27, 1925.   Body weight, 7.60 kilos.

Hourly period ending.	O_2	R.Q.	Average urinary N.	Total calories.		Blood sugar.	Blood CO_2.	Remarks.
				Calculated.	Found.			
	gm.		*gm. per hr.*			*per cent*	*vol. per cent*	
						0.065	48.5	Blood sample at 9.35
12.05 p.m.	3.34	0.77	0.065	10.99*	9.27			a.m. 78 mg. amytal
1.05 "	3.39	0.70		10.99*	10.55			per kilo at 9.38 a.m.
Averages .	3.37	0.73		10.99	9.91			
								7 units insulin per kilo
2.38 p.m.	3.16	0.75	0.065	10.36	10.72			at 1.08 p.m.
3.38 "	3.40	0.72		11.07	11.06			
4.38 "	3.49	0.75		11.45	11.56			
5.38 "	3.88	0.70		12.64	12.51	0.028	46.2	Blood sample at 5.40
Averages .				11.38	11.46			p.m.

* Average of two hourly periods.

Two experiments on an Airedale mongrel, one of which is given in Table V, form the third group.   In both of these experiments the respiratory quotient after giving insulin was constant for the 4 hours of observation but was at a higher level than during the preinsulin hours.   In both cases the level rose from 0.72 before insulin to about 0.78 after its injection.   There was no increase in heat production after administration of the hormone (Table V).

TABLE V.

Effect of Insulin on Metabolism of Dog 34 under Amytal Anesthesia.
May 15, 1925.   Body weight,   13 kilos.

Hourly period ending.	O_2	R.Q.	Average urinary N.	Total calories. Calculated.	Total calories. Found.	Body temperature.	Blood sugar.	Blood CO_2.	Remarks.
	gm.		*gm. per hr.*			*°C.*	*per cent*	*vol. per cent*	
						37.32*	0.076	51.0	75 mg. amytal per kilo at 9.15 a.m. Blood sample at 10.15 a.m.
12.15 p.m.	5.56	0.73	0.140	18.08	17.90	36.86			
1.15 "	5.97	0.71		19.42	16.99	36.38			
Averages.	5.77	0.72		18.75	17.45				
						36.30*			5 units insulin per kilo at 1.18 p.m.
2.43 p.m.	5.37	0.76	0.140	17.60	18.87	36.11			
3.43 "	5.37	0.78		17.79	16.08	35.70			
4.43 "	4.92	0.78		16.16	16.91	35.38			
5.43 "	5.35	0.79		17.67	15.80	35.11	0.037	50.6	Blood sample at 5.50 p.m.
Averages.				17.30	16.92				

* Body temperature at beginning of hour; other values at end of hour.

Blood and Urine Experiments.

In the data on the respiration experiments, Tables I to V, the results on the blood sugar and alkali reserve determinations are also tabulated.   In all of the respiration experiments with only one exception the carbon dioxide of the second blood sample which was taken 4 to 5 hours after the insulin injection was lower than the preinsulin figure.   The decrease varied between 0.3 and 5.9

volume per cent. The average of all the experiments was a decrease from 52.5 to 50.7 or 1.8 volumes per cent. When the carbon dioxide was followed in hourly periods it was found that the fall was greater than is indicated above and that the carbon dioxide value was returning to normal by the 4th or 5th hour after insulin. These data are given in Table VI.

The preinsulin control periods (Table VI and Chart 1) show that amytal anesthesia did not appreciably affect the alkali reserve of the blood. In all of the eight experiments in Table VI there

TABLE VI.

Effect of Insulin on Blood CO_2 in Dogs under Amytal Anethesia.

Dog No.	Experiment No.	Weight.	Amytal.	Insulin.	Preinsulin period.			Postinsulin period.					
					1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.
		kg.	mg. per kg.	units per kg.	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent	vol. per cent
30	3	9.2	65	6	48.4	47.9	48.0	48.7	40.2	42.0	41.8	44.3	48.1
30	5	9.0	65	6		48.5				43.0		47.5	
30	11	8.4	60	6			55.3		50.6	47.8			
34	2	12.2	70	4	42.9		43.1	42.6	41.7	33.6	38.2	38.4	39.4
35	1	8.8	55	4	53.1		51.8	52.1	41.8	40.5	46.0		
35	4	8.3	65	4			52.5		41.7		*		
36	6	16.1	60	5			49.1	47.4	47.2	43.6			
36	8	16.7	64	5	49.6			48.9	48.0	43.7			

* Dog died during the 4th hour.

was a distinct fall in the blood carbon dioxide after the insulin injection which did not start until after the 1st hour, reached its lowest point the 2nd or 3rd hour, and then slowly recovered. The extent of the decrease varied from 5.5 to 11.6 volumes per cent or from 11 to 22 per cent.

To show the time relationship between blood sugar and blood carbon dioxide changes a typical experiment (No. 2) on Dog 34, which was also used for the calorimeter work, is presented in Chart 1. The usual fall in blood sugar was found which probably began almost immediately. Chart 1 shows that the decrease in

blood sugar preceded that in carbon dioxide. The low point in the curves occurred at approximately the same time, about 3 hours after the insulin injection. The curves of Chart 1 are like those of a similar experiment (No. 3) on Dog 30 except that the delay before the decrease of the carbon dioxide curve is somewhat longer.
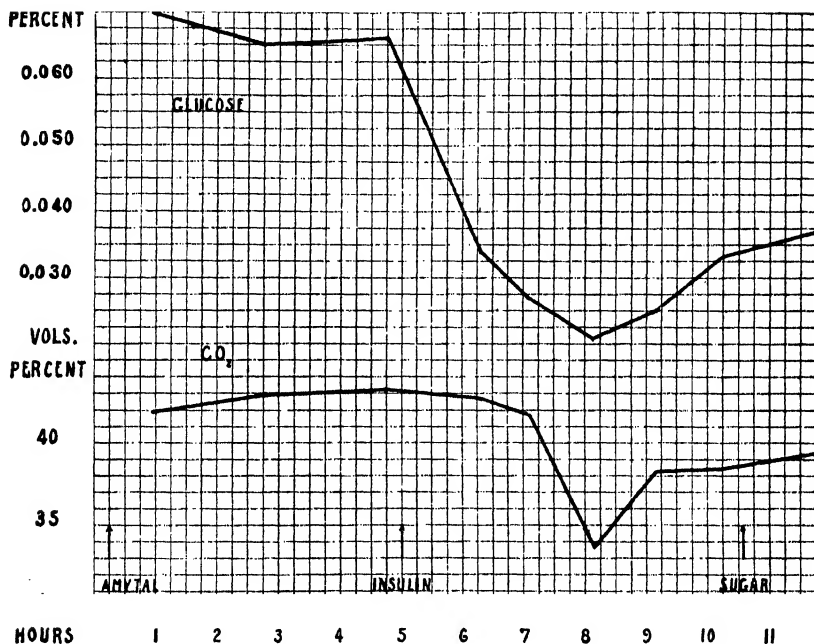
CHART 1. Dog 34, Experiment 2. Glucose and total carbon dioxide of blood; amytal, 70 mg. per kilo, insulin, 4 units per kilo, sugar, 50 gm. *per os.*

The work on the hourly urinary nitrogen samples taken simultaneously with the blood has since been extended and is being published separately (8).

DISCUSSION.

Our first group of experiments which show a rise in respiratory quotient during the 2nd hour after insulin is in agreement with the earlier work of Dickson, Eadie, Macleod, and Pember (9) on unanesthetized dogs and that of Hawley and Murlin on rabbits (4).

The shift in metabolism from fat to carbohydrate is well illustrated by the non-protein respiratory quotients of the experiment given in Table II.   These figures are 0.70 and 0.72 for the preliminary period and 0.71, 0.87, 0.72, and 0.70 for the insulin hours.   The ratio of heat produced from fat to that from carbohydrate was 99:1 during the preliminary period and the 1st postinsulin hour.   This was changed to a ratio of 43:57 during the 2nd hour after insulin. The variation was accomplished by a reduction in oxygen absorption rather than by an increased carbon dioxide production with no alteration in total metabolism.   Similarly in those cases illustrated by Table I the total heat production gradually rose after insulin, but during the 2nd hour the carbohydrate metabolism was relatively increased and the oxygen consumption relatively reduced.

In fasted animals there have not been reported previously results· such as we found in Group 2 in which there was no evidence of an increased carbohydrate metabolism.   In well fed rabbits with subconvulsive doses no change was noted by Chaikoff and Macleod (3) contrary to the earlier work of Dale and coworkers (10, 11) on eviscerated spinal cats.   In fasted rabbits the former investigators concluded that there was a rise in respiratory quotient.   In some cases it seems to us to be hardly beyond the experimental variation and is certainly not so great as that found by Hawley and Murlin.

In the third group (Table V) the stimulation of carbohydrate to replace fat metabolism extended over the whole 4 hours of observation and at a constant level.   In both experiments the preliminary non-protein respiratory quotients show that no carbohydrate was being burned.   After insulin from 15 to 30 per cent of the heat from the carbohydrate-fat mixture was due to the oxidation of carbohydrate.   As this type of reaction was only found in one animal it may be an individual variation.

Dickson and associates (9) reported no significant change in blood carbon dioxide after insulin, whereas Boothby and Weiss (12) found generally a decrease of 4 to 7 volumes per cent.   The latter investigators have calculated that the increased respiratory quotient after insulin might be due to a blowing off of carbon dioxide rather than an acceleration of carbohydrate metabolism.   It is noticeable in our results that the lowest point in the alkali reserve

of the blood appeared during the 2nd or 3rd hour, thus correspond-
ing to the time of the increased respiratory quotient when it oc-
curred.   However, in our experiments the fall in alkali reserve was
a reaction to insulin as regular in occurrence as the drop in blood
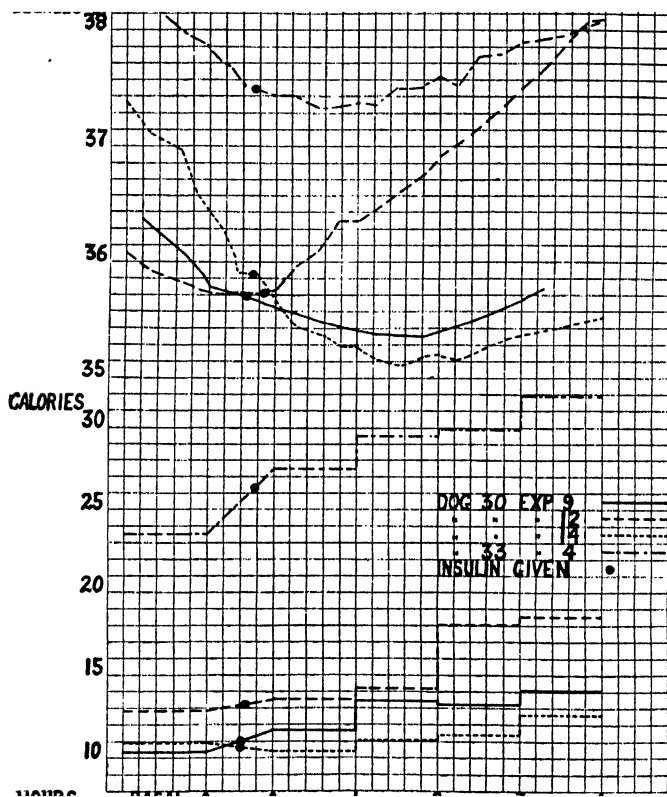
CHART 2.   The metabolism and rectal temperature of dogs before and
following the administration of insulin.   Amytal anesthetic used.

sugar, whereas the increased respiratory quotient during the 2nd
hour was found in only four out of ten experiments.   Likewise
there was no definite increase in the excreted carbon dioxide as
collected in the calorimeter to correspond to the blood changes.
It seems possible that the change in the alkali reserve of the blood

may be associated with the maintenance of osmotic equilibria between blood and tissues coincident with the decrease in blood sugar, for the fall in alkali reserve began in most of the experiments when the blood sugar reached a level of approximately 0.04 per cent.

The increase in heat production after insulin in some of our experiments was not in accord with the previous work with curare (5, 6). The control experiments on amytal (7) have shown that the body temperature fell slowly after its administration or remained at a constant subnormal level unless raised by shivering. Apparently in some instances the insulin provided the stimulus for an increased heat production which was followed by a rise in body temperature. The heat production and body temperature curves for four typical experiments are shown in Chart 2. The agreement between the calculated heat production and the direct calorimetry determinations is given in the tables (Nos. I to V). To what extent the subnormal body temperature caused by the amytal is responsible for the calorigenic effect of the insulin can only be conjectured. It is notable that in some cases with a lowered temperature there was no stimulation of heat production by the hormone.

SUMMARY.

The respiratory metabolism of fasting dogs under amytal anesthesia has been studied for 2 or 3 hours before and 4 hours after the injection of convulsive doses of insulin.

Three types of reaction to the hormone are noted as follows: four experiments in which there was no definite change in respiratory quotient, four showing a marked increase during the 2nd postinsulin hour, and two in which a rise in respiratory quotient extended over the 4 hours of observation. These variations in the reaction to insulin which were exhibited in different animals as well as in the same animal in different experiments may possibly be related to the glycogen content of the animal.

The acceleration in carbohydrate metabolism in some instances is merely a shift in the ratio of fat to carbohydrate burned with a corresponding reduction in oxygen consumption. In other cases it is associated with a rise in total metabolism.

Hourly determinations of the blood alkali reserve show a de-

crease in every experiment; hence it is not coincident with the rise in respiratory quotient. The fall in total carbon dioxide amounts to between 5.5 and 11.6 volumes per cent. The time relationship is pointed out between the changes in blood sugar and blood carbon dioxide.

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NUTRITIVE PROPERTIES OF THE MUNG BEAN.*

By V. G. HELLER.

(From the Department of Agricultural Chemical Research, Oklahoma Agricultural Experiment Station, Stillwater.)

The growing popularity of chop suey and other articles of diet which contain as their principal constituents the dry or germinated mung bean, lends an interest to the nutritional value of this bean. From a commercial standpoint, this interest is enhanced by the possibility of its growth in certain semiarid sections of this country where the short growing period makes the production of many of the popular articles of diet, for both man and animal, impossible.

The mung bean is probably a native of India, but its production is now common throughout all southern Asia and other tropical countries. Its growth in this country has not progressed far beyond the experimental stage, and its use as an article of diet for both man and animals has not been sufficiently extensive to lead to any definite conclusions.

The literature makes reference to a few reports in regard to certain chemical and nutritional properties, largely of the foreign grown bean. Embrey (1) refers to its use as an article of table diet in Asiatic countries. Santos (2) reports that it contains vitamin B, while Bowman and Yee (3) state that they prepared vitamin B crystals from this source. Johns and Waterman (4) extracted certain protein fractions and made a study of their nitrogen distribution. A further review of the literature failing to reveal any studies in connection with the nutritional value of the protein or vitamins of the seed, an attempt was made, using biological methods, to determine these properties. Any seed so rich in protein and capable of being grown in the short growing season of some of our hot, dry sections, if possessing a well balanced amino

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acid distribution and a good source of vitamins, would prove to be a valuable supplement to the diet often lacking in variety in these sections of our country.   Since the completion of this work an article by Tso (5) reports a study of its protein utilization.

<center>EXPERIMENTAL.</center>

In the nutritional tests, albino rats were chosen as experimental animals due to the well established growth curves in connection with deficiency disturbances as well as their short reproductive period.   It has been observed that the full test of a ration's deficiency is most clearly recognized in the response of the second and third generations.

The rats used were approximately 1 month of age, chosen from average sized litters.   The growth period, age of reproduction, number of young reared, as well as the percentage of young weaned of this particular strain, have been under observation over a period of several years.   In making tests the animals were housed in circular metallic cages, four or more animals to the cage, so selected as to be as similar in size, age, and litter origin as possible. The usual precautions in the preparation of pure rations, weekly weighings and examinations, and cleanliness were observed throughout the tests.

The mung beans used in these tests were solid green seed of a dwarf variety grown on the experimental plots of the Agronomy Department of this institution.   For all feeding experiments they were ground fine, and for chemical analysis further pulverized until the total quantity would pass through a 60 mesh sieve.   A proximate chemical analysis gave the following results:

	per cent		per cent
Protein.	23.31	Fat.	1.02
Water.	9.31	Fiber.	3.64
Nitrogen-free extract. . .	59.85	Ash.	2.87

A comparison of these figures with those of Embrey (1), reported in his studies of the Chinese mung bean, shows that the American grown seed is somewhat superior.   The protein content being of especial interest, a further chemical analysis was made to ascertain whether the nitrogen distribution was complete. For this study the method of Hamilton, Nevens, and Grindley (6) was followed with a few minor modifications.   The fractions so obtained are given in Table I.

Previous experiments carried out by numerous investigators have demonstrated that the proteins of a single cereal are usually not satisfactory for normal growth and reproduction unless supplemented with proteins or amino acids from other sources. This is due to the fact that certain amino acids are usually absent or deficient even though the total protein content is high. The protein content of the mung bean is high, as shown above, comparing favorably with that of the velvet bean. We were therefore interested in determining whether the amino acid distribution

TABLE I.

Distribution of Nitrogen in Mung Bean Hydrolysate.

Fraction.	Percentage of total nitrogen.
Total N in sample	3.73
Ether-alcohol-soluble N	None.
Alkali-insoluble N	4.71
N in hydrolyzed sample	93.80
" lost in hydrolysis	0.49
Amide N	6.41
Humin "	3.84
Cystine N.*	1.62
Histidine N.*	6.76
Arginine N.*	13.51
Lysine N.*	12.81
Total monoamino N	49.10
" diamino and cystine N	34.70
" N recovered	94.05
" " accounted for	98.76

* Corrected for solubility of phosphotungstic precipitates.

was more equitably represented than in other members of the bean family, a group possessing a high protein content but unfortunately usually a poor balance of amino acids.

Experiments were planned fully to check the character of this particular protein. The rations used in this study contained 60 per cent of the bean, representing approximately 14 per cent protein. Provisions were made that the proper amount of salts and vitamin should be added to supplement any deficiency other than amino acids, previous investigation having demonstrated that the ions sodium, calcium, and chlorine are usually limited in

most seed.   Vitamin A is also usually deficient in these seeds for optimum development.

A second set of experiments was planned by using the optimum protein level found in the preceding experiments, to investigate the vitamin content of the seeds by omitting first one and then another vitamin and observing the result to establish the vitamin content of the seed alone.

A third set of experiments was carried out to determine whether inorganic ions other than sodium, calcium, and chlorine might be deficient.   A detailed statement of these rations is shown in a condensed form in Table II.

Ration I aṣ indicated in Table II consisted of 60 per cent ground mung bean supplemented only with 1 per cent of sodium chloride

TABLE II.

Ration No............................	I	II	III	IV	V	VI	VII	VIII
Mung bean.................	60	60	60	60	93.5	92.5	80	80
Starch.....................	38	35	36.5	30			15	18
NaCl......................	1	1		1			1	1
CaCO₃.....................	1	1		1			1	1
Cod liver oil...............		3		3	3	3	3	0
Salt Mixture 185.*..........			3.5		3.5	3.5		
Extract of wheat embryo...					5			
Cystine....................						1		

* McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 63.

and a similar amount of calcium carbonate to which dextrin was added to make the entire up to 100 parts.   This practice permits the variation of various supplements without altering the amount of seed present.   Ration II adds a further supplement of vitamin A in the form of cod liver oil.   Ration III replaces sodium chloride and calcium carbonate with 3.5 per cent of McCollum's Salt Mixture 185, while Ration IV contains both.   Ration V contains yeast as a source of vitamin B, a deficiency not to be expected in a seed fed at this level.   The other rations carried higher levels of the bean protein to ascertain whether certain amino acids, evidently somewhat deficient in a 60 per cent level, might be adequately provided in a larger percentage of seed.   Cystine has many times proved to be the limiting factor in many rations, and

for this reason this amino acid was added to Ration VI. The most characteristic results of growth, reproduction, and rearing of young, produced by these rations, have been consolidated and are presented in Table III.

An investigation of Table III indicates that 14 per cent of protein from the single source as used in Ration I gave continued growth to maturity; the mothers gave birth to young, many of which were weaned. The growth even of the first generation was somewhat retarded, it is true, but this condition has been obtained with practically all rations containing protein from only one variety of seed.

To determine whether this result might be due to vitamin or salt deficiency, it is necessary to examine the results of other

TABLE III.

Ration No.	No. of males.	No. of females.	No. of litters.	No. of young.	No. that lived.	No. that died.	No. consumed.	Growth.
II	2	2	2	10	3	7	6	+++
I	2	2	2	11	11	0	0	+++
III	1	3	2	11	6	5	0	+++
IV	3	1	2	11	6	5	5	+++
V	3	1	1	8	7	1	0	+++
VI	1	2	2	10	10	0	0	++++
VII	2	3	3	18	16	2	0	+++
VIII	2	3	4	21	20	1	0	+++

++++ represents normal growth of young in the growing colony.

rations. Ration II, containing vitamin A in the form of cod liver oil, did not prove to be superior to Ration I, containing no such supplement, indicating that this bean is superior to most of our cereals, in that vitamin A is present in amounts necessary for normal growth. Ration III, having a vitamin B supplement, gave no better results. This was to be anticipated, as 60 per cent of most seeds contains an adequate amount of vitamin B for normal development. (It had previously been demonstrated by Honeywell and Steenbock (7) that the beans, while dry, were devoid of vitamin C but became an excellent source of that substance upon germinating.) The addition of a complete salt mixture showed no decided advantage. It then becomes evident

that while the bean is superior to many seeds, and especially most beans, it is either lacking in certain amino acids, or else they are present in such small amount that 60 per cent of the bean furnishes insufficient amount for optimum maintenance.   Ration V, which contains 93.5 per cent of the bean (21.5 per cent protein), while somewhat better in its results, is still lacking in its ability to produce normal development.   When cystine was added, the ration was improved as evidenced in the superior maintenance of the young, for with this ration reproduction was obtained to the third generation.   The somewhat retarded growth and lack of smoothness of fur marked the ration as being somewhat inferior to the mixed ration fed the stock animals.   The data do indicate that the protein is superior to that of many members of the bean family. Its vitamin content is superior to that of most cereals, in that vitamin A is more adequately provided.

TABLE IV.

Ration No.	Bean, 80 per cent.	Cod liver oil.	Salts.	Starch.
X	Raw.	3	3.5	13.5
XI	Heated 1 hr.	3	3.5	13.5
XII	" 2 hrs.	3	3.5	13.5
XIII	" 3 "	3	3.5	13.5

Effect of Cooking.

Inasmuch as beans for human consumption are usually cooked before they are eaten, further experiments were planned to determine the effect of heating on the utilization of the protein. In these cases the beans were autoclaved at 15 pounds pressure for 1, 2, and 3 hours, and then dried.   Vitamin A was added, because heating at these temperatures would undoubtedly injure this constituent.   Table IV indicates the compositions of rations used in these determinations.

Ration X, which was practically a repetition of ration VII gave similar results.   Heating for 1 hour seemed to indicate a slight improvement of utilization.   Ration XIII in which the seed had been heated for 3 hours was somewhat less satisfactory in promoting growth than either the raw or less heated lots. This is an observation previously noted by us in regard to heating

other foods. A short heating period may aid, but long exposure seems to be injurious to the nutritive properties of the protein. This decrease in nutritive value is not to be accounted for by vitamin destruction, as vitamin A was added to all rations, and 3 per cent of yeast incorporated into Rations XII and XIII during the last 4 weeks gave no evidence of accelerating the rate of growth.

Seasonal Variation of Mung Bean.

Unfortunately, the seeds of this plant do not mature uniformly, and consequently many of the riper seeds shell off before the unripe are ready for harvest. Naturally, this loss would reduce the

TABLE V.

Variety.	Age.	Ash.	Protein.	Ether extract.	Fiber.	Non-filterable extract.
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Standard............	91	9.77	21.82	2.66	22.98	42.83
"	98	8.44	21.54	2.41	24.00	43.49
"	112	8.32	19.71	2.56	27.04	42.27
Dwarf..............	91	8.16	16.83	2.44	22.44	50.00
"	98	9.05	16.99	2.95	21.13	49.88
"	112	9.17	18.92	2.87	20.65	48.39

value of the crop. For this reason, it was suggested that possibly the most economical method of handling would be to harvest the plant at some premature stage. The percentage of protein on a dry weight basis does not change much in the last days of growth. The beans after drying could be threshed and the plants remaining used as fodder for animals. With this in mind, an approximate analysis of the whole plant at various stages of growth was made, to determine the most desirable time of harvesting. Table V contains the analysis of two varieties of the mung bean plant, at different stages of growth, expressed as per cent of total dry matter. The result indicates that after growth and podding have taken place no important changes occur, and harvesting may be governed by other conditions.

CONCLUSION AND SUMMARY.

1. The mung bean is developed quickly in a hot dry climate and can be raised in the semiarid sections of our country.

2. Its protein content is high, and, as indicated by chemical analysis the nitrogen distribution is fair.

3. Nutritional tests show that rats can grow to maturity with protein from this single source. Growth is somewhat below normal, and reproduction is limited.

4. Vitamin A is present in amounts greater than is to be found `n many seeds.

5. A plentiful supply of vitamin B is present when the bean composes 60 per cent of the ration, and sodium, calcium, and chlorine seem to complete the mineral requirements.

6. A limited cooking seems to aid the nutritional value, whiie extensive cooking becomes detrimental.

7. The uneven ripening of the bean is detrimental but data obtained show that the entire plant may be harvested green any time after the pods are filled.

8. The indications are that the mung is a superior type of bean from a nutritional standpoint, but not adequate as a sole source of protein.

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THE NORMAL PIGMENT OF THE URINE.*

I. THE RELATIONSHIP OF URINARY PIGMENT OUTPUT TO DIET AND METABOLISM.

BY DAVID L. DRABKIN.†

(*From the Laboratory of Physiological Chemistry, Yale University, New Haven.*)

CONTENTS.

INTRODUCTION.

The diagnosis of disease solely through the inspection of the urine, in which the deviation of the color from the normal amber tint played the dominant rôle, was condemned as early as the sixteenth century (1). Surprisingly, however, the idea of the clinical importance of the color of the urine has persisted to the present day. Physicians still practice a medieval empiricism by qualitatively describing the tint of urine specimens in a space especially allocated to this purpose upon most bedside charts. Although the subject of urinary color has been amply discussed, the lack of definite information concerning this phenomenon, which is a part of daily experience, is disclosed by the unsatisfactory and, sometimes, misleading statements in the most recent text-books of physiological chemistry (2).

HISTORICAL.

The literature upon the coloring matter of the urine began in 1800 with the publication of Proust's pioneer attempt to identify the pigment chemically. The researches of Proust were rescued from oblivion by Thudichum (1864), who named the urinary pigment "urochrome." Since the latter's contribution numerous investigators have enlarged the literature upon this subject to almost unwieldy proportions, so that the critical discussion of many interesting papers (3–16) must be reserved for a later article. The contributions upon the relation of diet to the output of urochrome are few in number and are practically eclipsed by the chemical studies. They merit discussion, however, due to their pertinence to the present paper.

Forestus (1) has credited Gabriel de Taregua, a follower of Avicenna (eleventh century), with the idea that the quantity and quality of the diet may alter the color of the urine. Browiński and Dombrowski (17), utilizing the reducing property of urochrome to determine it directly by titrating the iodine liberated by the pigment from iodic acid, have stated that the quantity of urochrome was increased in typhoid, pneumonia, and some hepatic cirrhoses, was below normal in nephritis, and higher on a pure meat than on a pure milk diet. They have commented upon the last observation as

follows: "La molécule albuminoïde contient probablement deux groupes chromogènes, dont l'un rich en soufre, fournit les matériaux de structure des pigments mélaniques et de l'urochrome, derivés des transformations des substances albuminoïdes." The method employed by these investigators is obviously unreliable for the quantitative determination of the urochrome output and the observations were too limited in number to permit generalizations. Pelkan (18) attempted to study the influence of various proteins upon the excretion of the normal urinary pigment. His conclusions unfortunately do not keep within the narrow limits of his actual experimental data: "All evidence points to the fact that urochrome, lactochrome [(13)], and protochrome are identical and, at least to a large extent, are derived from food proteins." This generalization, however interesting, appears to the writer to lack foundation in fact and to be completely vitiated by the following statement of Pelkan's method, which, in the light of all past researches on this subject must be considered wrong: "A 24 hour sample of urine was collected and a small portion of it filtered and compared in the Duboscq colorimeter with an arbitrary standard of 3.2 mg. of Bismarck brown and 8 mg. of Echtgelb Y [Klemperer's standard (19)] in 1 liter of water. The color corresponded closely to that of the normal urine, and a value of one unit per cc. was assigned to it. *Another determination was made by adding 5 cc. of a 20 per cent solution of lead acetate to 25 cc. of the urine*[1] and comparing the filtrate with another arbitrary standard of 3 mg. of Echtgelb Y in 1 liter of water. In this manner two determinations were made of each 24 hour sample, one on the total color of the filtered urine, and another on the urine from which *the proteins and such colors as urobilin and uroerythrin had been largely removed by precipitation with lead acetate, and which contained, therefore, urochrome as the principal coloring matter.*[1] In calculating the color units of the precipitated urine, due allowance was made for the dilution with lead acetate." That Pelkan lost a great deal of his urochrome by precipitation with lead acetate one can hardly doubt. Indeed, it is remarkable that all of his samples apparently retained some measurable color and none was completely decolorized by such treatment. A critical examination of Pelkan's data shows, furthermore, that the loss of urochrome by the method of analysis and not an actual reduction in the output of pigment was, in all probability, responsible for the conclusions. Such an error in procedure can only be ascribed to the confusion of the recent literature upon the normal urinary pigment, caused, mainly, by a superfluity of reports upon the so called "urochromogen reaction" of Weisz (14) and the ambiguous statements of the latter concerning his methods of analysis. To this day not the slightest acceptable proof has appeared in support of the idea that urochromogen is truly unoxidized urochrome.

The only other paper upon the relation of diet to the excretion of the urinary pigment is the citation of a single experiment by Roaf (20). Work-

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[1] The italics are ours.

ing upon guinea pigs and considering "the experiments so easy of repetition that full experimental details need not be given," this investigator postulated the exogenous origin of urochrome from chlorophyll. There is some evidence that, under a high chlorophyll regimen, porphyrin-like substances may appear in the urine (Kortschagin, Hofstetter, Godinko, and Kitahara (21)), so that in estimating urochrome output, under such conditions, the urine must first be freed from these adventitious pigments. Roaf's work is therefore inconclusive.

The most recent papers (15, 16) upon the chemistry of urochrome have not materially advanced our knowledge of this pigment. From a mass of hypothetical and contradictory statements, the reader can extract only two points of general agreement, the facts that urochrome is mainly responsible for the color of normal urine, and that, upon treatment with sulfuric acid, the urinary pigment is transformed into a melanin-like substance (3, 4, 11, 22). The chemistry and physiology of the normal pigment of the urine still remain obscure.

Plan of Problem.

The development of successful methods for the maintenance of animals on so called synthetic diets, composed in large part of purified food materials that can be modified at will, has enhanced the possibility of a more effective investigation of the urinary pigment without the interference of extraneous coloring matters. Possible complications represented by such common food components as carrots (13), green vegetables (21), or red meat can thus be averted.

Accordingly an attempt was made to study the normal variations in urinary pigment output under conditions in which all extraneous pigments were eliminated; and, subsequently a study of the influence of various factors—dietary, functional—was instituted. It became necessary at the outset to develop a suitable method for the quantitative estimation of urine color. With the technique described below it has been possible to make an elaborate study of the changes in pigment output under a wide range of experimental conditions in man and various species.

Methods.

Colorimetric Standard for Quantitative Estimation of the Pigment.

In the absence of information as to the chemical make-up of urochrome, an adequate colorimetric method, employing as an empirical standard a suitable mixture of readily obtainable dye-

stuffs, seemed best to meet the needs of the investigation. A standard was accordingly made up in the following way: 200 cc. of 95 per cent ethyl alcohol containing 0.3 gm. of basic alizarin (trocken, Kahlbaum) were slowly heated to boiling, with constant stirring. The solution was filtered while hot into a liter cylinder, which had been previously moistened with alcohol. To obtain the last drops of filtrate the fluted filter paper was gently loosened from the funnel. The filtrate, wine-red in color and having a volume of about 196 cc., was made up to 250 cc. with 0.5 N HCl, the color thereby changing to yellow-ochre. The solution was next diluted to 1 liter with distilled water. A light, flocculent orange precipitate formed at this point. Upon filtration a pale, fluorescent, yellow-green solution was obtained. It represents a saturated, weakly alcoholic, acid solution of alizarin.

A 0.005 per cent aqueous solution of aniline orange was next prepared. For convenience and to insure accuracy it was prepared in great excess: 100 mg. of dry aniline orange were dissolved in 200 cc. of distilled water by heating to boiling and, after filtration, were diluted with distilled water to 2 liters. The color is golden yellow in this dilution. The addition of more than small amounts of this aniline orange solution to the alizarin solution, both of which are yellow in color, was found to give rise to solutions of a distinct and beautiful red. Thus, by adding the proper small quantity of the aniline orange to the alizarin solution, the slightly too greenish tint of the latter was modified by the production of a faint tinge of golden pink. The proper proportion of aniline orange to alizarin was found to be 1 part to 80. In the preparation of the standard it was found necessary to be very cautious in the exact quantitative preparation of the aniline orange solution and in the use of exact quantities in making the mixture, slight excesses of aniline orange resulting in marked deviations of the color of the final standard solution.

By following the above directions the color standard has been duplicated a number of times. The color has remained unaltered during a period of 2 years and may therefore be considered relatively permanent. It simulates closely the color of urine and it possesses, too, a slight greenish fluorescence. When in comparable states of concentration the urines of man, dogs, and rats were almost invariably found to agree in tint. The same standard

could therefore be employed in the case of all three species according to the following empirical rules, which were found useful.

(*a*). The urines of dogs were, in most cases, diluted to approximately twice their original volume. The diluted daily volumes thus varied, in this species, from 200 to about 600 cc. Dilution was, of course, not resorted to in cases of diuresis, etc. Human and rat urines, on the other hand, were colorimetrically determined undiluted, in most instances.

(*b*). All urines were subjected to preliminary filtration, those of dogs through a triple layer of filter paper and those of man through either a double or triple layer. Those samples in which the turbidity persisted after filtration and occasional samples with a pinkish tint, which made accurate determination difficult, were rejected. In some instances urine samples were found with colored uric acid sediments. These were gently warmed before filtration to insure against the possible loss of pigment.

(*c*). In the case of the urines from adult dogs and man, with the Duboscq type of colorimeter, 20 mm. were found to be the ideal depth for the standard; in the case of rat urines and the pigment extract from human milk whey (later studied) a depth of 10 mm. seemed preferable. Preliminary experiments also established the fact that strictly proportional readings could be obtained with the standard at 10, 20, and 25 mm. and with urines of varying dilution. The determinations were made upon 24 hour specimens and were expressed in empirical units, the value of 1 unit being assigned to the pigment content of 100 cc. of urine when it just matched the standard color already described. The output of urinary pigment per day could be easily calculated by the formula, $U = \dfrac{V \times R^1}{100 \times R^2}$, where U represents the units of pigment, V the volume of urine in cc., R^1 the depth of the standard, and R^2 the reading (depth) of the unknown.

Accuracy and Value of the Colorimetric Method for Determining the Normal Urinary Pigment.

In the absence of reliable guarantees of the purity of urochrome preparations, an exact index of the accuracy of the above method for the determination of the normal urinary pigment could not be obtained. The general trend of the data has indicated that

the error is of the order of ±5 per cent. This figure is of much lower magnitude than the experimentally produced differences in pigment output.

In making use of the colorimetric method a serious question arose. Was it justifiable to consider the colorimetric determination an index of the quantity of a single pigment, the *normal urinary pigment?* Or, was the determination merely an expression of the cumulative color effect due to the presence of many substances which made contributions of color to the whole? From a critical consideration of the literature, especially the work of Garrod (9), there was every reason to believe that substances of the type of urobilin and hematoporphyrin are negligible in their contributions to normal urinary color. It was considered advisable, however, to fortify personal views by the preliminary examination of about 100 human and canine normal urine specimens. The simple plan pursued was the following. After filtration, the pigment content of the urine specimen was determined. To remove such substances as urobilin, uroerythrin, and most of the hematoporphyrin (if present) the urine was treated by a modified Garrod (9) method, *viz.*: It was rendered alkaline with a very small amount of CaO and saturated with $(NH_4)_2SO_4$. The precipitate was filtered off. It was found important to extract the precipitate several times with water in order to insure against the loss (as great as 30 per cent) of water-soluble pigment which was carried down with the precipitate (probably mechanically). The aqueous extracts (from the precipitate) were combined, concentrated *in vacuo*, and added to the filtrate. The quantity of pigment in this filtrate was determined. The pigment values of the original and treated urine samples were compared. In many instances, too, both samples were read against each other in the colorimeter. In the many observations taken the differences in the colorimetric readings of original urines and the corresponding treated samples (*i.e.* with extraneous pigments such as urobilin removed) were negligible. In the case of normal human and canine urines (collected to avoid fecal contamination, etc.) the colorimetric determination of untreated, whole urine is a true index of the quantity of the normal urinary pigment, urochrome.

As a control measure during the course of the entire research, the possible presence of extraneous pigments in unusual or abnor-

mal amounts was eliminated by chemical tests, etc. This precaution was especially applied in those cases where there was any reason to suspect the presence of abnormal pigments. Cases in point, for example, are those in which diets containing hemoglobin and chlorophyll were used. To insure against the presence of porphyrins in these instances (as indicated by the work of Hofstetter, Godinko, and Kitahara (21)) the pigment readings were made upon urine specimens treated in the way which has been described except that more lime was employed in the precipitation. The dog is said to have a "low biliary threshold" and high fat diets are thought to stimulate bile flow. Such ideas may have been at the basis for the origin, in some circles, of an unfounded belief as to the existence of varying amounts of bilirubin in normal canine urines. This pigment has been sought for in vain by suitable tests and chloroform extraction in an extensive number of normal dog urine specimens and cannot play a rôle in the results obtained in these studies.

In the study of clinical material, on the other hand, the presence of pathological pigments such as bilirubin in cases of jaundice and hematoporphyrin in cases of sulfonal poisoning (Quincke (23)), etc., must be borne in mind to insure the correct interpretation of one's findings. It is a matter of common knowledge (frequently forgotten by students of the urinary pigments) that certain dyestuffs of exogenous origin can abnormally color the urine. Forestus (1), writing more than 300 years ago, was fully aware of the effect of rhubarb, saffron, caffia, Dantzick beer, etc., and considered the urine to be truly chameleonic. The appearance of water-soluble dyestuffs, such as methylene blue, in unchanged form and of some of the fat-soluble dyes, in conjugated form, in the urine, following their administration *per os* or by injection, is well known. The conditions of carotinuria (13) and, possibly, anthocyanuria (24) and the abnormal coloration of the urine after the administration of certain drugs (23) are also well recognized. In using hospitalized patients as subjects for the study of the influence of various pathological conditions upon the metabolism of the normal urinary pigment these various exogenous influences upon the color of the urine must be eliminated.

Basal and Other Diets Employed. Controlled Conditions of the Experiments.

The conditions of the various experiments, most of which were upon healthy, female, mongrel terriers of 4 to 10 kilos body weight, were completely controlled by the employment of a strict metabolism technique, in which the special feature was the diet. To obtain a food mixture devoid of color and at the same time complete in all known dietary essentials is not simple. One need only to be reminded, for example, that fat-soluble pigments such as the xanthophylls and carotin constantly accompany (probably mainly because of similar solubilities) fat-soluble vitamin A. The basal

TABLE I.
Basal, Colorless, Diet (Kilo Unit).

	Amount.	Calories.	Percentage.
	gm.		
Coagulated commercial egg albumen (14.5 per cent N).....................	5.50	22.0	33.7
Sucrose...............................	6.50	26.0	39.9
Lard................................	3.17	28.5	19.4 ⎫ 21.4
Cod liver oil.........................	0.33	3.0	2.0 ⎭
Bone ash............................	0.60		3.7
Salt mixture.*.......................	0.20		1.2
Total............................	16.30	79.5	99.9

* Karr or Cowgill salt mixture (25).

diet chosen was only relatively colorless—a small amount of pigment being possibly contributed by the cod liver oil and Vitavose (a vitamin B product derived from wheat embryo, tested as to potency by Cowgill, Deuel, and Smith (25)) and was, with minor modifications, essentially that described by Cowgill (25). The quantity of bone ash was somewhat increased in amount. The higher ash level gave ideal results in respect to laxation, diarrhea being avoided, the feces being solid and dry, and, at the same time, constipation not resulting. At first commercial albumin was employed as the source of protein. To avoid possible diarrhea it had to be coagulated, dried, and finely ground before being incorporated into the food mixture, which on the basis of a kilo unit had the composition given in Table I.

This diet was so made that 0.8 gm. of N was furnished per kilo, 40 per cent of the calories was furnished by fat, and a total of approximately 80 calories was fed per kilo of weight. Experience has shown that small dogs require more of the diet and must be fed at a higher calorie level than large animals. The exact amount to be fed to maintain the dog in excellent condition (in N balance, etc.) was adjusted through trial. For the details of the scientific basis and preparation of the diet the reader is referred to Cowgill's (25) original paper. The difficulty of preparing the albumin diet and its greater cost led, early in the study, to the substitution of casein for albumin in the above food mixture and, unless otherwise stated, this is the standard diet employed. To insure against vitamin B deficiency 0.6 gm. of Vitavose per kilo of body weight was added to the daily ration. It was found best to mix the vitamin powder in with the food. The special advantage of the diet used was the ease with which it could be modified to suit experimental purposes, so that other mixtures were readily obtained for use in comparative experiments in the study of the influence of changes in diet upon the normal pigment of the urine. Thus, Diet B was a high protein mixture, containing 50 per cent by weight of casein, Diet C was one in which 15 per cent by weight of chlorophyll (prepared from alfalfa by the method of Willstätter (26)) was incorporated, Diet D contained 23 per cent by weight of hemoglobin (prepared in crude, powdered form from sheep blood), Diet E was the standard diet mixed with an aqueous solution of the whey pigment from 5 quarts of cow's milk (prepared by the method of Palmer and Cooledge (13)), and Diet F was a mixture of the standard diet and a purified aqueous extract of the normal urinary pigment from a 6 day urine collection from the same dog to which it was fed. (The preparation was by a modified Garrod (9) method.) Other diets employed besides the standard and those obtained from it by modification were the Cowgill (25) meat residue diet, essentially the same type as the standard, a dog biscuit diet, and a raw meat diet. In the case of the dog biscuit diet in which there is a tendency to diarrhea the special precaution was taken to obtain the urine solely by catheterization (several times a day) and thus avoid fecal contamination. It was thought worth while also to study the influence of changing from a colorless to a colored diet upon man. Proteins, in the colorless diet, were supplied by coagulated egg

albumen, skimmed milk (to avoid fat pigment) and Cream of Wheat. Other articles in this diet were cane sugar, skinned potatoes, rice, table salt, Crisco, and water. A small quantity of cod liver oil was taken. The choice of a diet is small when one restricts it to colorless nutrients. The differences in pigment output upon this diet, used for 5 days, and various restaurant diets were studied.

The effects of short period fasting, of the administration of acids and alkalies and of calorigenic agents such as thyroxin, epinephrine, and phlorhizin were next studied. That considerable increments in metabolism can be produced by certain chemical derivatives is well recognized,—thyroxin (Kendall; Hirsch and Blumenfeld; Kunde; Hunt; Plummer and Boothby; Boothby, Slosse, and Sandiford (27)), epinephrine (Lusk and Riche; Ringer; Sandiford; Boothby and Sandiford; Hitchcock (28)), and phlorhizin (Lusk (29)). The thyroxin (Squibb) was administered to dogs intravenously (in dilute alkaline solution) in doses of 2 to 3 mg. on each of 3 to 5 consecutive days, so that a total of about 10 mg. was administered by the step-up method (see Kendall (27)). The epinephrine was given subcutaneously in doses of 1 mg. or 1 cc. of 1:1000 solution per kilo. It was found necessary to control the results here obtained by the administration of large amounts of water by stomach tube in order to learn the effect of simple diuresis upon the pigment output. 1 gm. of phlorhizin in sterile olive oil per day was administered by Coolen's method, subcutaneously. Alkali, $NaHCO_3$, was given to dogs by stomach tube in doses of 150 cc. of a 5 per cent solution or 0.75 gm. per kilo of body weight; acid, HCl, in doses of 1 gm. per kilo, a 0.2 N (approximately 0.7 per cent) solution being used. This amount, 10.3 gm. in one dog, 5.6 gm. in another, was quite in excess of that given by Walter (30). The acid was administered until the appearance of the first signs of increased pulmonary ventilation. The administration of acids and alkalies and even of different types of foods, which are known to affect the reaction of urine (Henderson and Palmer; Blatherwick (31)), necessitated the consideration of a possible source of error in this method of attacking the problem; namely, the influence of the pH of urine upon the intensity of the color. By use of appropriate indicators (bromphenol blue and thymol blue), it was found that between pH 4.6

and 9.6 the reaction is unrelated to the color. At a pH somewhat below 9.6 phosphates precipitated and at a pH between 3 and 4.6 urates were thrown down. In abnormal, very highly acid urines (pH below 3), however, a pinkish tint developed making it impossible to obtain accurate readings with the color standard employed in these studies.

One of the minor procedures employed was an attempt to produce myxedema in one of the dogs by the operative removal of the thyroid glands, leaving the two upper parathyroids intact. This was done to observe whether an effect opposite to that of calorigenic stimulation would produce the opposite response in reference to the normal urinary pigment metabolism. The effect of amytal anesthesia (32) was observed in one case.

The experimental studies upon dogs were supplemented by studies upon the pigment output of a large group of normal men (medical students), upon a smaller but entirely corresponding group of women, and upon a number of pathological cases.

Collection of Urine Specimens.

In the case of adult dogs complete 24 hour urine specimens were assured by obtaining by sterile catheterization the last traces of urine retained in the bladder and washing the bladder out with sterile water. In the case of rats, urine was collected in 4 day periods by the special rat metabolism technique of Levine and Smith (33). In these cases the factor of fecal contamination could not be accurately controlled so that the results are much less reliable than those upon dogs. In the case of puppies, too, where catheterization was impossible, the urines were collected in weekly periods, under toluene. That the factor of slight ammoniacal decomposition does not influence the color of urine *per se* was determined by the colorimetric comparison of urine samples, collected in periods of varying duration, from 1 to 5 days, from the same animal. The readings obtained from the different samples were entirely proportional. In one case air was bubbled through unpreserved urine. The color readings, corrected for evaporation, did not change appreciably during the first 2 days, although phosphates were precipitated. On the 4th day the urine was rendered valueless for colorimetric determination by the de-

velopment of molds, etc. Human urine specimens, preserved under toluene, and kept in well stoppered bottles in the cold were in excellent condition even after 2 weeks, which included, in some cases, a trip in the mail from Detroit to New Haven. The rule was to determine specimens of dog urines daily.

In the collection of the urines of dogs, the most important factor to control and the greatest possible source of error was found to be fecal contamination. Only a very small amount of highly pigmented feces is needed to alter completely the color of a urine specimen. This factor of error can only be eliminated by the strictest of precautions. The procedure adopted was as follows: The metabolism cages were kept thoroughly clean at all times. The excretory habits of the individual dogs were studied and the feces were, as far as possible, removed soon after they were formed. This involved constant supervision. The standard diet employed was a great help in this connection. The animals were fed about 10 a.m. after catheterization and before the employment of special procedures, such as drug administration, in some of the studies. The dogs usually had one bowel movement daily, in the afternoon. In most instances the feces, of dogs upon the Cowgill diet, had the virtue of being hard, dry, and almost colorless. The water content of the feces varied somewhat with the fluid intakes of individual animals. In three of the dogs, kept under a metabolism régime for periods of from 1 to nearly 2 years, the amount of obvious pigment in the feces seemed apparently to vary in a type of cycle: For 2, 3, or 4 days the feces were relatively pale or even devoid of color and were firm and dry. Then, for 1 or 2 days the fecal material was very much altered, being semisoft and very darkly pigmented. There was no exact order to this cycle of events and whether it is true of all dogs or has any special physiological significance is uncertain. But, at any rate, it must be borne in mind that, in the dogs under observation, the opportunities for the urine to become contaminated by fecal pigments was much greater on some days than on others. Thus, neglect to remove promptly the feces when formed could readily have resulted in abnormally high urine pigment values from time to time.

In the case of the present investigation it was decided to eliminate the customary washing of the pan. The limit of accuracy of the colorimetric method is such that the loss of even 2 per cent

of the urine (probably 1 per cent is the most that can be lost by sticking to the pan) cannot materially influence the result. On the other hand, the recovery of traces of urine by washing the pan would be more than offset by the introduction of contaminants into the urine sample, such as tiny particles of scattered food, hair, and skin scales and tiny amounts of feces, which sometimes are scattered to the corners of the pan and miss being collected. The slight scattering of food is unavoidable but is minimal with the Cowgill diet, which is quickly and completely eaten by the dog. In the case of diets such as the dog biscuit diet, with which loose bowel movements were common, the fecal contamination of the urine was avoided by catheterizing the animal periodically, so that the voluntary voiding of the bladder did not take place. A specimen of urine obtained solely by catheter, is, in a way, the ideal specimen. That laxation *per se* does not influence urine pigment output was seen by feeding a standard diet in which bran had been incorporated. The pigment output was the same upon this diet as upon the basic ration.

By following the above precautions, most of the canine urine specimens, after filtration, were perfectly clear.

Experimental Results.

Pigment Output, under Controlled Conditions, in the Normal, Adult Organism.

In eleven adult dogs (four of which, C, S, N, and L, were observed over long periods of consecutive days—1 to 2 years), by using the basal regimen which has been described, the output of urinary pigment, after a short period of adjustment to the diet (as in Dog N, Chart 1), was constant from day to day. Similar results were obtained with normal human subjects, although the dietary conditions of the experiment could be controlled only approximately in these cases. The maximum variations in pigment output upon the colorless diet, which are recorded in Table II, average 4.0 per cent.

Relation of Diet to Output of Normal Urinary Pigment.

Radical changes in the regimen from the basal colorless diet to food mixtures, most of which were highly colored, were found to

result in only negligible deviations in the quantity of pigment in the urine. The special diets were substituted for the basal ration for several days and the pigment outputs during 7 consecutive days were determined. That the pigment output is essentially independent of the diet is indicated both by the slight deviations from the basal diet level and by the fact that, upon some of the special diets, the variations are not uniformly in the same direction. In the experiment on man the variation of $+16.6$ per cent

TABLE II.

Basal (Colorless) Ration.

Data compiled from daily analyses during 1 week.

	Weight.	Pigment output (in units).		Variation of maximum from minimum.
		Minimum.	Maximum.	
	kg.			*per cent*
Dog J................	26.0	10.2	10.6	3.9
" X................	18.6	8.1	8.3	2.5
" B1...............	12.7	8.0	8.7	8.7
" B2...............	11.0	7.1	7.2	1.4
" N................	10.4	6.5	6.6	1.5
" W2*.............	9.5	5.8	5.8	0.0
" W1..............	9.0	4.0	4.2	5.0
" L...............	8.0	6.4	6.6	3.1
" F2...............	6.8	4.2	4.5	7.1
" S................	6.8	5.0	5.1	2.0
" C................	5.2	3.8	4.0	5.3
Man.................	67.3	11.2	12.0	7.1
Average...				4.0

* Only two determinations.

in changing from the special colorless diet (which has been described) to a mixed diet is of a somewhat higher order than that observed for dogs. In the light of all the experiments, however, it does not appear significant (Table III).

Effect of Fasting, Administration of Acids and Alkalies, Calorigenic Stimulation, and Diuresis upon the Amount of Pigment Eliminated in Urine.

The attention of the writer was drawn to the study of the influence of other factors besides diet upon the output of the urinary

TABLE III.

Effect of Special Dietary Components.

Data compiled from daily analyses during 1 week.

| Food component. | Weight. | Pigment output (in units). | | Gain or loss in pigment output. |
		Basal diet.	Extreme change on experimental diet.	
	kg.			*per cent*
Dog N. Diet B, 50 per cent protein.	10.4	6.5	6.5	0.0
Meat diet.		6.5	6.5	0.0
Bran "		6.5	6.5	0.0
Dog biscuit diet.		6.4	6.5	+1.6
Dog W1. Diet B.	9.0	4.1	4.3	+4.8
Dog L. Diet C, 15 per cent chlorophyll.	8.0	5.1	5.3	+3.9
" D, 23 " " hemoglobin.		5.3	5.1	−3.8
" F, urochrome.		5.5	5.9	+7.3
Meat diet.		6.5	6.2	−4.6
Dog S. Diet C.	6.8	5.0	5.4	+8.0
" D.		5.0	5.1	+2.0
" . E, milk whey pigment.		5.2	5.2	0.0
" F.		5.2	5.2	0.0
Meat residue diet.		5.1	5.1	0.0
Bran diet.		5.0	5.0	0.0
Dog C. Diet B.	5.2	3.8	4.2	+10.5
" C.		3.9	3.6	−7.7
" D.		3.9	4.2	+7.7
" E.		3.7	3.9	+5.4
" F.		4.0	4.3	+7.5
Meat residue diet.		3.9	3.9	0.0
" diet.		3.9	3.7	−5.1
Dog biscuit diet.		4.1	4.0	−2.4
Man.* Mixed restaurant diet.	67.3	12.0	14.0	+16.6

* Basal diet, special colorless diet.

pigment by the chance observation in one of the students of a 22.5 per cent reduction in pigment (18.4 to 14.2 units), when the diet was changed from an acid to a basic nature (by the introduction of liberal amounts of fruit salad, etc.). A markedly altered pigment output in dogs was found to result from fasting, the administration of acid, and, especially from the augmented metabolism, produced by stimulation with such calorigenic agents as thyroxin, epinephrine, and phlorhizin. The results of the various experiments are graphically shown in Chart 1 and the maximum variations in per cent of the pigment output from the level upon the basal diet are given in Table IV.

Fasting.—An average, maximum increase of 46.4 per cent in urinary pigment output occurred by fasting the animals for periods of 1, 2, and 3 days. The increase was greater from day to day during the short fasting period. Whether a level would be reached in longer fasting periods was not determined. The administration of sodium bicarbonate during the fasting period to a certain extent offset the increase (Table IV, Dog N, 9.1 to 6.7 units of pigment, a reduction of 26.4 per cent).

Acids and Alkalies.—An average, maximum increase of 34.6 per cent in pigment output followed the administration of hydrochloric acid. The effect of alkali was much less marked, being negligible in the normal animal, an average reduction of only 8.6 per cent. This was probably in part due to the use of relatively small amounts of alkali as vomiting resulted from larger quantities, preventing effective administration *per os*. That the reaction of the urine itself did not account for these results has already been pointed out.

Metabolic Stimulation or Depression.—The striking increases in pigment output, which resulted from the administration of metabolic stimulants to animals are summarized in Table IV and Chart 1. Qualitatively, interesting differences in the pigment output occurred with the three agents employed. The increase with epinephrine was sudden and marked, rapidly disappearing in 1 to 2 days after administration. With thyroxin, on the other hand, the increase was gradual to a high level, which was well maintained for about 2 weeks, gradually wearing off. As might be expected, the persistence of effect with thyroxin was even more marked when given to Dog L which had its thyroids previously

removed. The very high figures obtained with phlorhizin are probably in some measure accounted for by a difficulty in color comparison. With the appearance of sugar a marked deep greenish blue fluorescence, not due to urobilin, was present in these urines. What this interesting effect was due to could not be definitely determined. It may be related to the presence of urinary sugar as its persistence was coincident with that of the latter.

Following thyroidectomy, although the animal was an adult, signs suggestive of myxedema (Dog L, Chart 1) began to appear. There was an apparent reduction in activity, increase in weight, loss of hair (not due to mange), and a turgidity of the skin. Accompanying these phenomena there was a marked fall in pigment output, 6.4 to 3.0 units, 53.1 per cent reduction. After 2 months, however, the pigment output began to return towards normal, 3.0 to 5.2 units, and the symptoms disappeared. An exploratory operation was performed 8 months after the thyroidectomy in the belief that regenerated thyroid tissue would be found. This, however, could not be demonstrated at the site of the operation, which presented two visibly enlarged, isolated parathyroids. Some suspicious tissue was removed for histological examination, but proved to be merely lymphoid in character.

Diuresis.—An increased urinary volume almost invariably occurred simultaneously with increased pigment output. This relative diuresis effect was, in a general way, proportional to the increase in pigment and was noticeable especially after the administration of acids and metabolic stimulation. To eliminate the possibility that an increase in pigment output may result from merely a flushing action upon the kidney, diuresis was evoked in Dogs C, S, N, and L through the administration by stomach tube of large quantities of water. Although the volume of urine was doubled and trebled in these instances, the pigment output remained undisturbed. It is worthy of note that, in a general way, the volume output of urine in the different species appeared to be proportional to the mass of the animal: A rat, weighing 0.2 kilo, had a urinary volume of approximately 5 cc. per day, a dog of 5 kilos eliminated approximately 125 cc., and a man of 70 kilos, 1500 to 1700 cc. per day.

TABLE IV.

Effect of Fasting and of Administration of Acids, Thyroxin, Etc.

Data compiled from daily analyses during 1 week.

	Type of experiment.	Weight.	Pigment output (in units).		Gain or loss in pigment output.
			Basal diet.	Extreme change due to experimental procedure.	
		kg.			*per cent*
Dog N.	Fasting (3 days).	10.4	6.0	9.2	+53.3
	"		5.9	9.1	+54.2
	Alkali administration.*		9.1	6.7	−26.4
	Acid "		6.6	8.5	+28.8
	Epinephrine "		6.5	11.5	+77.0
Dog L.	Acid administration.	8.0	6.5	9.1	+40.0
	Phlorhizin "		6.7	19.3	+188.0
	Thyroxin "		6.5	8.1	+24.6
	" " †		3.3	7.1	+115.1
	Thyroidectomy (upper parathyroids retained).		6.4	3.0	−53.1
Dog S.	Alkali administration.	6.8	5.2	4.8	−7.7
	Epinephrine "		3.9	4.9	+25.6
	Phlorhizin "		5.2	9.9	+90.4
	Thyroxin "		5.1	7.5	+47.1
Dog F2.	Fasting (2 days).	6.8	4.3	6.3	+46.5
Dog C.	Fasting (2 days).	5.2	4.6	6.2	+34.8
	"		4.4	6.3	+43.2
	Acid administration.		3.7	5.0	+35.1
	Epinephrine "		3.8	7.2	+89.5
	Thyroxin "		4.1	6.4	+56.1
	" "		3.9	5.9	+51.3
Man.	Alkali administration.	67.3	11.6	10.5	−9.5

* Alkali administered during fasting.

† Thyroxin administered after removal of thyroid glands.

Effect of Anesthesia, Strychnine, and Irradiation with Ultra-Violet Light.

Two animals, which were anesthetized with amytal (isoamylethylbarbituric acid), eliminated urines with a normal pigment content. Strychnine administration likewise failed to influence the urinary pigment output. Irradiation by light from a mercury vapor lamp, on the other hand, was found in one animal to cause
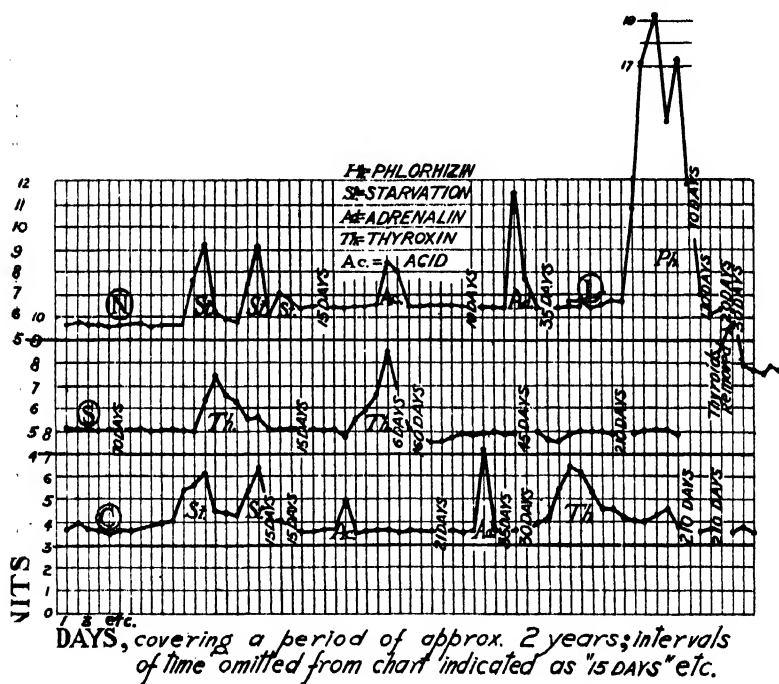
CHART 1.

an increased pigment excretion. How constant the latter phenomenon is cannot, of course, be judged on the basis of a single observation. It should be pointed out, however, that irradiation with ultra-violet light cannot have a bearing upon any of the experiments reported in this paper. The animals were kept in metabolism cages and the light conditions in the room were constant.

Comparative Data with Different Species and Pigment Output in Growing Dogs.

Table V presents data which permit the comparison of the pigment output in the urine of twenty-three animals, representing three different species (rat, dog, and man), the individuals of each group varying widely in size. The quantity of pigment output per day varied from 21.9 units for a large adult man to 0.41 unit for a small rat. A comparison of the output of pigment upon a per kilo of body weight basis discloses a relationship typified by the following: Rat R1, weighing 184 gm., eliminated daily in the urine 2.23 units of pigment per kilo of body weight; Puppy S4, 1.34 kilos in weight, eliminated 1.71 units of normal urinary pigment upon the same basis; Dog C, 5.2 kilos body weight, excreted 0.73 unit; the human infant of approximately the same weight as Dog C put out very nearly the same quantity of pigment, 0.81 unit, and the adult 77.3 kilos man excreted only 0.23 unit of pigment per kilo of body weight. This relationship can be more strikingly expressed by saying that, per kilo, a rat puts out in the urine 8.0 times the quantity of pigment eliminated by an adult man, 3.0 times that of a medium sized adult dog, 2.7 times that of an infant, and 1.3 times more than a puppy. These findings naturally led to the recalculation of the data in relation to the surface area. With the exception of the data for puppies and rats, representative of the period of rapid growth, the figures for the pigment output per square meter of body surface in the different individuals of the three species cluster together within remarkably narrow limits (Table V, last column).

Chart 2 graphically depicts the pigment output data upon four puppies during a period of 10 weeks of rapid growth. The chart is self-explanatory. While the total pigment elimination per day increased rapidly with increase in size, per kilo of body weight the values remained quite constant, showing a tendency, indeed, to a gradual decline with growth.

Plotting the urinary pigment excretion (Table V, Column 5) against the surface area (Table V, Column 4) yielded, by inspection, a straight line, the slant of which indicates that the pigment output is approximately 11.0 units per square meter of body surface. Using this graph, it was found that, given the surface area,

the pigment output of a normal organism could be predicted within fairly narrow limits. It was so employed together with the re-

<div align="center">

TABLE V.

Relation of Pigment Output to Size of Individual.

</div>

Comparative data for three different species.

Species.	Weight.	Height.	Surface area.*	Pigment output per day	Pigment per kilo of body weight.	Pigment per sq. meter of body surface.
	kg.	*cm.*	*sq.m.*	*units*	*units*	*units*
Man 3...............	77.30	182.5	1.980	21.9	0.28	11.1
" 5...............	59.40	170.0	1.690	17.7	0.30	10.5
" 2...............	58.10	167.5	1.650	18.4	0.32	11.2
" 1...............	39.00	150.0	1.290	14.7	0.38	11.4
" 4...............	6.00		0.406	4.9	0.81	12.1
Dog J..............	26.00		0.992	10.4	0.40	10.5
" X..............	18.60		0.793	8.3	0.44	10.5
" B1.............	12.70		0.615	8.3	0.65	·13.5
" B2.............	11.00		0.559	7.2	0.65	12.9
" N..............	10.40		0.538	6.5	0.62	12.1
" W2.............	9.50		0.507	5.8	0.61	11.4
" W1.............	9.00		0.489	4.2	0.45	8.6
" L1.............	8.00		0 452	6.4	0.80	14.1
" L2.†...........	7.90		0.448	5.2	0.66	11.6
" F2.............	6.80		0.406	4.4	0.65	10.8
" S...............	6.80		0.406	5.0	0.74	12.3
" C...............	5.20		0.339	3.8	0.73	11.2
" S6.‡...........	3.33		0.274	4.4	1.32	16.1
" S5.‡...........	1.30		0.146	2.2	1.56	15.1
" S4.‡...........	1.34		0.149	2.1	1.71	14.1
Rat R1.‡............	0.184		0.0294	0.41	2.23	13.9
" R2.‡............	0.227		0.0339	0.57	2.51	16.8
" R3.‡............	0.234		0.0346	0.47	2.01	13.6
" R4.‡............	0.240		0.0351	0.58	2.42	16.5 ·

* Determination of surface area: adult men by Du Bois height-weight chart; human infant by formula $12.3\,W^{\frac{2}{3}}$; adult dogs, by formula $11.3\,W^{\frac{2}{3}}$; puppies, by formula $12.3\,W^{\frac{2}{3}}$; rats, by formula $9.1\,W^{\frac{2}{3}}$.

† Same dog as L1, approximately 7 months after thyroidectomy.

‡ Growing animals.

sults obtained in a "normal distribution curve" as an index to the normal pigment output in the interpretation of clinical data.

Diurnal Variation in Pigment Output; Relation to Meals.

The pigment output during the day, the period of active metabolism, was found, in a number of experiments to be greater than during the night. Dog C eliminated in the urine 2.2 units of pigment, Dog N 3.6 units, and a human subject 10.2 units during the day period. The pigment outputs of the respective night urine samples were 1.6, 1.9, and 4.3 units. By changing the feeding time from the day to the night (giving the diet at midnight) it was hoped to gain some information concerning the influence of the meal, the process of digestion, etc., upon the form of the daily
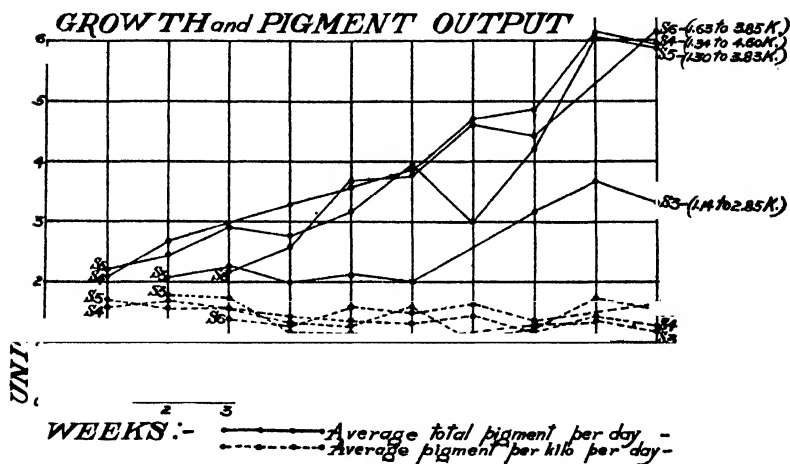
CHART 2.

pigment output curve. Such a change in feeding time caused Dog C to excrete a somewhat greater amount of pigment during the night (2.2 units) than in the day period (1.9 units), a reversal, indeed, of the previous figures. The withdrawal of food at the habitual time, however, made the animal restless. This may account for the small increase in total pigment output. In the absence of ideal conditions the writer does not feel justified in interpreting the results.

Daily pigment output curves for two human subjects showed two periods of high output, one in the late morning (about 10 to 11 a. m.), and the other, still higher, in the late afternoon (about 5 p. m.).

Clinical Data.

Normal Distribution Curve for Pigment Output in Man.

In order properly to interpret clinical data it was essential to establish an index of normal pigment excretion.   The data obtained from a class of male students at the Yale School of Medicine, when plotted, yielded a normal distribution curve (Chart 3).   The average output of pigment per day was 19.0 units.   One of the students, whose output was, for an unknown reason, abnormally high at first (40 units) later had consistent values at the average

NORMAL DISTRIBUTION CURVE

Average male output = 19.0 Units
or 9.9 Units/SqM
Average female output = 12.7 Units
or 8.2 Units/SqM

Number in each group

Units of Pigment

●——●——● MALE MEDICAL STUDENTS (FRESHMEN)
▬——◆——▬ FEMALE STUDENTS
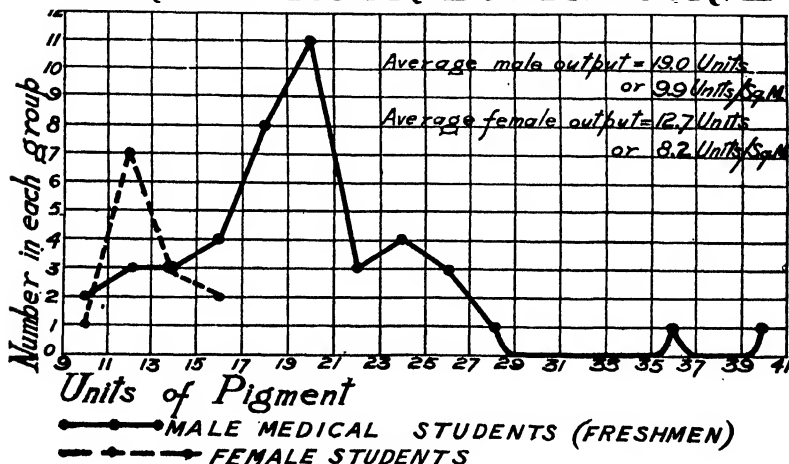
CHART 3.

normal level for males.   The writer was fortunate in obtaining the cooperation of an exactly comparable, though smaller, group of young women at the Merrill-Palmer School, Detroit.   The normal distribution curve for this group, Chart 3, yielded an average of 12.7 units of urinary pigment per day, or 17 per cent below that of the male group (upon a square meter of body surface basis). In determining an approximate value for normal in the clinical data upon female subjects this factor was made use of as a correction for the pigment output values obtained from the chart constructed from the data in Columns 4 and 5, Table V (see p. 463).

Pigment Output in Orientals.

Since data of observations upon the lower metabolism of Orientals have appeared (34), it seemed of interest to study such a group in regard to urinary pigment output. Thus far, only a limited number of individuals has been studied so that conclusions cannot be drawn. A Chinese woman (W. Y. H.) with a surface area of 1.3 square meters and a basal metabolic rate of 34.6 calories per square meter per hour had a pigment output of 12.0 units per day or 2 per cent above the calculated normal figure for a white woman of the same surface area (see Chart 3). A Japanese woman (F. M.) with a surface area of 1.22 square meters and a basal metabolism of 32.8 calories per square meter per hour eliminated 8.7

DAYS ——— Mr. C., Severe Graves Disease, ⌖-Day of Complete Thyroidectomy.
- - - - Mrs. M., Normal Pregnancy, ⊘-7ᵗʰ month; ⊘-8ᵗʰ month; ⊘-preparturient period
⌖-Day of Delivery; ⊘-5 weeks post partum.
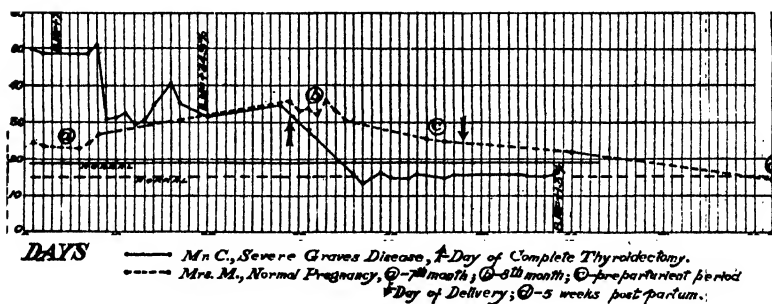
CHART 4.

units of pigment or 21 per cent below the calculated value. A male (S) of the latter race, whose surface area was 1.68 square meters and whose expected pigment output on the basis of the normal white male was 18.7 units excreted 16.1 units of pigment per day or 14 per cent below the calculated level.

Pigment Output in Pregnancy, Exophthalmic Goiter, Diabetes, Nephritis, and Typhoid.

Chart 4 represents two carefully studied clinical cases. Mrs. M., a normal pregnant woman, showed a very much increased pigment output during the later months of pregnancy. The pigment output fell slightly just before parturition but was still far above normal limits. 5 weeks after delivery, although lactation

TABLE VI.

Effect of Disease.

Disease entity, clinical diagnosis.	Date.	Weight.	Height.	Surface area.*	Metabolism increase or decrease.	Pigment output per day.		
						Calculated normal figure.†	Actual figure.	Deviation from normal.
		kg.	*cm.*	*sq.m.*	*per cent*	*units*	*units*	*per cent*
Graves' disease.								
Mr. C........	May 10–11, 1925.	45.0		1.430	+75.0	16.0	49.6	+210.0
	June 1–2, 1925.	55.0		1.634	+39.0	18.0	32.0	+77.7
	" 14–15, 1925.	58.0		1.693	+1.3‡	18.7	16.7	−10.6
Mr. U........	Dec. 29–30, 1925.	37.7	179.0	1.437	+65.0	16.0	32.9	+105.6
	Jan. 4–5, 1926.	39.1		1.458	+55.0	16.2	25.6	+58.0
Mrs. M........	May 3–4, 1926.	51.4	167.5	1.560	+72.6	14.2	18.3	+28.8
	May 22–23, 1926.§						40.5	+187.2
	June 4–5, 1926.						23.2	+64.5
Mr. L........	Jan. 1–2, 1926.	57.7	165.8	1.625	+73.0	17.8	36.2	+103.3
Mrs. H........	Dec. 30–31, 1925.	53.6		1.548	+27.0	14.1	22.4	+58.8
Miss B........	Jan. 4–5, 1926.		153.8	1.497	+25.0	13.6	26.7	+96.3
Miss S........	Jan. 2–3, 1926.	24.3	137.5	0.985	−4.6‡	9.1	13.6	+49.4
	" 6–7, 1926.				+15.0		10.0	+9.9
Mrs. E........	Dec. 29–30, 1925.	53.0		1.564	+48.0	14.3	23.0	+60.8
Arteriosclerosis, emphysema, and heart disease.								
Mr. H........	Dec. 30–31, 1925.	60.0		1.685	−3.0	18.4	42.1	+128.8

Diabetes mellitus.							
Mr. C.	June 3–4, 1925.				19.0	19.6	+3.1
	" 17–18, 1925.||					23.2	+22.1
Mrs. C.	Nov. 9–10, 1925.				12.7	16.4	+29.1
Nephritis.							
Mrs. K.	Oct. 14–15, 1925.				12.7	12.8	+0.8
Uremia.							
Mr. H.¶	Oct. 9–10, 1925.				19.0	5.9	−69.0
Mrs. G.	" 14–15, 1925.				12.7	8.0	−37.0
Polycythemia vera, treated with phenylhydrazine.							
Mrs. B.	Oct. 17–18, 1925.				12.7	58.8	+363.0
Typhoid.							
Miss C.	June 23–24, 1925.	24.0	128.5	0.940	8.6	11.5	+33.7
Mr. P.	" 26–27, 1925.	38.0	122.5	1.100	12.1	13.7	+13.2

* Surface area, determined by the Meeh-Rubner formula, surface $= 11.3W^{\frac{2}{3}}$ in the case of Mr. C. with Graves' disease and by means of the Du Bois height-weight chart in all the other cases.

† The figures for the normal outputs of urinary pigment were derived from the surface area by means of the chart previously mentioned (p. 463) or by the formula, pigment output $= 11.0 \times$ surface area. In those instances in which surface area data were lacking, the values so obtained were reduced by 17 per cent. In the cases of female patients, the only normal values available to the writer for the purpose of comparison were those of 19.0 units for males and 12.7 units for females, the average values obtained from the respective normal distribution curves, Chart 3. The figures for the deviation from normal therefore more nearly approximate the true state in those cases where the surface area was calculated.

‡ Metabolism (basal) after thyroidectomy.

§ 1 day after the ligation of the thyroid vessels; the specimen of urine of May 3 to 4 was probably incomplete.

\|\| Mild diabetic, requiring 25 units of insulin; specimen of June 3 to 4 was sugar-free; sugar appeared in specimen of June 17 to 18.

¶ Terminal uremia, 1 day before death; the other case, Mrs. G., early uremia.

was at its height, the urinary pigment values fell to almost exactly the previously calculated, normal level. The quantity of lacto-chrome eliminated in the milk was determined by extracting this pigment by a modified Garrod-Palmer (13) method. 640 cc. of milk, representing a 24 hour secretion volume, were found by the above procedure to contain 1.4 units of pigment. Upon the same day 14.2 units of pigment were excreted in 2025 cc. of urine. Thus, even if the whey pigment proves in ultimate analysis to be identical with the normal pigment of the urine, its quantity in human milk appears to be too small to alter significantly the total pigment excretion.

The second graph shown in Chart 4 was plotted from the data upon a severe case of Graves' disease. The patient's metabolic rate and clinical progress under various therapeutic measures were found to be closely reflected in the day to day variations in urinary pigment output. After complete thyroidectomy the pigment fell to somewhat below the calculated normal level.

Table VI is a résumé of the pigment output determinations upon a small number of clinical cases. The amount of urinary pigment eliminated in patients with exophthalmic goiter was consistently high and the variations in pigment output from day to day were sometimes great. The increase in basal metabolic rate was not always proportional to the increment in pigment excretion.

The pigment output in a number of diabetics, whose urines were sugar-free, was found to be essentially normal. With the appearance of sugar in the urine, although the color was qualitatively pale (due to dilution), the total pigment output, in two cases, was actually increased. The increment in pigment, however, was of a much lower order of magnitude than in the Graves' disease cases.

In one case of nephritis, uncomplicated by uremia, the pigment output did not deviate from the calculated level for normal. In two individuals with uremia the pigment in the urine was found to be below normal, especially in the terminal stage of the disease. The amount of pigment eliminated was, however, in the cases observed, probably entirely accounted for by the reduction in the volume of urine eliminated and not by a specific retention of pigment. This seems plausible because the concentration of pigment

in the urine excreted (some of it recovered by catheterization, Mr. H., Table VI) was normal.

The pigment output level in two typhoid patients was appreciably above normal, but the increments were not as great as might have been expected from the other cases studied.

In an individual with advanced arteriosclerosis and heart disease the pigment output was very high, but the basal metabolic rate was normal.

DISCUSSION.

The observations of the present investigation, which indicate that the animal organism, under controlled conditions, eliminates a constant quantity of normal urinary pigment from day to day and that the pigment output is essentially independent of the diet, were contrary to personal expectations. In this connection reference may be made to the recent papers of Hédon (35) and Benedict (35) upon the constancy of basal metabolism in the dog and man. It now appears reasonable that urochrome is a chemical entity, which belongs to the aristocracy of biochemical substances, the endogenous products.

Tissue breakdown or an intensification of the cellular life process may be assumed to be the cause of the marked increases in pigment output, which were observed to follow the administration of acids, short periods of fasting, and, especially, the stimulation of metabolism with such calorigenic agents as thyroxin, epinephrine, and phlorhizin, of the increased pigment elimination in high fevers, such as typhoid, and in cases of Graves' disease, and, finally, of the augmented urinary pigment output in the later months of pregnancy. In prolonged fasting an increase in basal metabolism has been observed (Kunde (36)), but the specific, immediate influence at the basis of an increased pigment elimination in the present fasting experiments is probably the same as that in the case of the administration of acids, an acidosis. This opinion is supported by the fact that the administration of alkalies in fasting tended partly to offset the pigment increase.

The possibility that the urinary pigment may be produced or may originate in the kidneys (Becher (16)) appears fanciful. The fact that the volume of urine was found to vary, in a general way, with the quantity of pigment seems to lend strength to the idea;

which, however, is completely refuted by the fact that diuresis *per se* (produced by forcing fluids) did not increase pigment output. Flushing out the kidneys by an increased volume of fluid could not, therefore, have been the cause of increased pigment output in the case of stimulation by acids and calorigenic agents, etc.

The result, obtained by plotting the normal urinary pigment values of rats, puppies, adult dogs, infants, and human adults of different sizes against their surface areas (p. 463), indicating that the quantity of pigment eliminated is a straight line function of the surface area, is noteworthy. Although the number of individuals (Table V) in each of the three species plotted for comparison was necessarily limited by the scope of this investigation, the results can hardly be fortuitous. The significance of the facts may be brought out by the following statements: Under controlled conditions, in the different animals studied, the quantity of urinary coloring matter per square meter of surface is fairly constant. From the graph of the pigment output per day against the surface area (Column 7, Table V, and p. 463) either the pigment or the surface can be quite accurately foretold when the value of the other is known. The basal metabolism, in the normal organism, being directly proportional to the surface area, may be expressed, within the limits of accuracy of this study, as so many units of normal urinary pigment. The ability to prophecy the pigment output from the surface area, as has been done repeatedly during the course of the research, affords pragmatic evidence of the relationship of pigment, surface, and metabolism. With the discovery of the chemical identity of urochrome and in the event of the confirmation of these findings by a more exact technique, basal metabolism may be expressed as so many mg. of pigment. It would represent a true measurement of cellular metabolism. So far as the writer is aware, the relationships which have been observed for urochrome are unique in biochemical literature and may prove of the same general significance as Rubner's (37) surface area law. That the method of estimating the quantity of urinary pigment employed in this paper is delicate and accurate enough, even in its present colorimetric form, to furnish data of interest and value is made evident by an examination of the data. For example, the difference in metabolism of human males and females was brought out (Chart 3) and was of the same order of magnitude as the values

arrived at by measurements of the heat production (see Lusk's "The elements of the science of nutrition" for basal metabolism data). Another example in point is furnished by the observations upon rats and puppies (Chart 2 and Table V). The pigment output, as is the case with the heat production, was relatively higher during the period of rapid growth than in adult life. The pigment excretion per day increased with growth, but the output per kilo of body weight showed a tendency to decline slowly during this period. The writer places less reliance upon the results obtained from growing animals than upon the completely controlled experiments with adult dogs, where fecal contamination, etc., was entirely eliminated, but, due to the consistency of the findings upon the four puppies and an equal number of growing rats, he is satisfied that the results represent a close approximation of the truth. The value of the present method for the determination of urinary pigment output is also attested by the fact that it has served as a delicate and reliable index of variation in metabolic rate and of clinical progress, under various therapeutic measures, of a number of hospitalized cases, such as, for example, Mr. C. with severe exophthalmic goiter and Mrs. M., a normal woman in pregnancy (Chart 4 and Table VI).

In interpreting the meaning of abnormally high values for the output of the normal urinary pigment (urochrome) in pathological cases it must not be assumed that the finding of increased pigment output is always associated with increased heat production (basal metabolism). Most of the cases, studied thus far (Table VI), were chosen because they were known to be accompanied by the phenomenon of elevated metabolism. In one case at least, that of advanced arteriosclerosis, emphysema, and heart disease, a marked increase in urinary pigment was observed in the presence of a normal basal metabolic rate. There are very probably other types of pathologic conditions in which high pigment output values may be found unassociated with disturbances in heat production. The exact causes of increased urochrome elimination remain undefined. Tissue breakdown or metabolism in the broad sense seems definitely concerned. The fact that individuals with advanced heart disease and normal metabolism (in the specific sense of normal heat production, measured calorimetrically) may eliminate abnormal quantities of pigment does not, in the writer's opinion,

alter the significance of the relationship of basal metabolism and urinary pigment output in Graves' disease, etc.

No special significance can be attached to the abnormally high elimination of urinary pigment in a case of polycythemia vera, for the patient was undergoing heroic treatment with phenylhydrazine and pigment values were, unfortunately, not obtained before the administration of the drug. The formation of a brownish color in phenylhydrazine upon exposure to air is a well known phenomenon and there was reason to suspect that in this case at least a good part of the increment in color could be accounted for by the elimination of phenylhydrazine or its derivatives in the urine, although there was not sufficient material definitely to establish the fact. This is an illustration of the caution that is necessary in studying urines in which medicinal agents or their decomposition products may be eliminated.

The relationships which have been described for urochrome call for the comparison of the urinary pigment with another endogenous product, creatinine, the determination of which Folin (38) believed to afford the most clear cut value of normal tissue metabolism. A critical review of the creatine and creatinine literature (see Myers (39)) discloses that an exact explanation of the genesis of creatinine in the organism has thus far met with insurmountable obstacles. Creatine is definitely not an end-product; its utilization is somehow bound up with carbohydrate metabolism, as was originally proved by Mendel and Rose (39). The evidence as to the constancy of the heat production (calories)-creatinine ratio (Palmer, Means, and Gamble (39)) appears inconclusive and in dogs, which have proved to be the best animals for careful calorimetric work, the coefficient of creatinine excretion (possibly due to method) is much less definite than in the case of man (Fonteyne and Ingelbrecht (39)). With the development of a more accurate technique for creatinine determination in the dog, the inquiry as to a possible parallelism of creatinine and urochrome output may be in order. At present, however, it appears that the two substances travel along somewhat different metabolic paths.

Viewed in the light of the possibility that urochrome is a complex containing neutral sulfur in organic combination (Dombrowski (11), Weisz (14), and Fischer (15)), the observation of the constancy of urinary pigment output has added significance,

for it has been found that the neutral sulfur elimination in urine is quite constant (Folin (38)). In recent years, neutral sulfur has been endowed with special virtues in biological economy (40).

The origin of the urinary pigment from the blood pigment, hemoglobin, still remains as a possibility, although its genesis from tissue proteins or the protein component of the respiratory pigment appears more likely. On the other hand, preformed tissue pigments must also be considered as a very possible source of urochrome. Such pigments are the muscle pigment, myohemoglobin (Kennedy and Whipple; Whipple (41)), the interesting tissue pigment, distinct from myohemoglobin, cytochrome (Keilin (41)), and the respiratory ferment (Atmungsferment) of yeast (Warburg (41)).

Lactochrome, the pigment of milk whey (Palmer and Cooledge (13)) and a pigment extracted from egg albumen (by the writer), while of interest, can, at best, only be said to be urochrome-like substances. The question of the distribution in nature of pigments identical with the normal urinary pigment and the related problem of the possible presence of urochrome precursors in tissues and fluids besides urine (the histogenesis of urochrome) will be undertaken with profit only when new methods for isolating the pigment have been discovered and products free from adventitious contaminants have been obtained. The writer is now engaged in an effort in this direction.

SUMMARY AND CONCLUSIONS.

A technique for the study of the metabolism of the normal urinary pigment under completely controlled conditions has been developed and thoroughly tested as to its reliability.

The results obtained indicated that, in some respects, the coloring principle of urine is biochemically unique. The output of pigment in the urine by a normal organism was found to be a constant quantity from day to day. The quantity of pigment eliminated was essentially independent of the regimen, which, starting with a practically colorless standard diet, was subjected to radical changes. On the other hand, the pigment output was markedly augmented by fasting, by the administration of acids, and, above all, through the stimulation of the metabolism by means of several calorigenic agents. A fluctuation of the quantity

of urinary pigment in the opposite direction (*i.e.* a decrease) was observed after the administration of alkalies and following the surgical removal of the thyroid glands (in the dog). The observations were extended to include disturbances of metabolism in man. In cases of Graves' disease (exophthalmic goiter) the urinary pigment output was abnormally high and, in one case, studied from day to day, it was found to vary with the patient's metabolic rate and served as an index of clinical progress under varied therapeutic measures. The studies, furthermore, were given broader significance by a comparison of the results obtained in the rat, dog, and man, which indicated that the figures for the urinary pigment output of the three species (including individuals of different sizes in each) when plotted against the surface area gave a straight line. The observations are too limited in scope and number to justify the final pronouncement of a principle, but, such as they are, they point to the fact that the quantity of urinary pigment per unit of surface is a biological constant.

The data which have been presented permit the conclusion that the normal pigment of the urine is an endogenous product. It is eliminated in quantities which are probably proportional to the intensity of the endogenous metabolism, of which it may, therefore, prove to be a reliable index.

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THE NORMAL PIGMENT OF THE URINE.

II. THE RELATIONSHIP OF THE BASAL METABOLISM TO THE OUTPUT OF THE NORMAL URINARY PIGMENT.*

BY DAVID L. DRABKIN.

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.)†

The experimental results of a comprehensive study of the normal urinary pigment, urochrome, have led to the conclusion (1) that the quantity of pigment output is essentially independent of the diet and directly proportional to the basal metabolism (as calculated from the surface area) in the normal rat, dog, and man. It was found possible to increase markedly the output of the urinary pigment by stimulating the metabolism of dogs with such agents as thyroxin. Furthermore, a number of individuals with exophthalmic goiter were found to have an abnormally high pigment excretion. In view of these observations it was desirable to make a further study of pathological individuals, especially those with diseases characterized by the phenomenon of abnormal heat production. Accordingly, a new series of clinical cases, including nine patients with Graves' disease (exophthalmic goiter), one with leucemia, and three with lobar pneumonia, has been intensively studied, under controlled conditions. The study was carried out during the fall and winter of 1926–27. An attempt was made,

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TABLE I.

Pathological condition.	Surface area.*	Basal metabolism.	Pigment output per day.			Remarks.
			Calculated normal value.	Actual value.	Deviation from normal.	
	sq.m.	*per cent*	*units*	*units*	*per cent*	
Graves' disease, Mrs. E. K.	1.512	+53	13.8	21.0	+52.2	Patient resistant to therapy.
	1.515	+59	13.9	22.8	+64.0	
	1.520	+47	14.0	18.1	+29.2	
	1.552	+73	14.2	27.4	+93.0	
	1.550	+62	14.2	25.5	+79.5	
Miss F. M.		+29	14.2	18.2	+28.1	Mild case. Death at operation. Status lymphaticus found at autopsy.
	1.549	+20	14.2	17.7	+24.6	
			14.2	14.9	+4.9†	
Mrs. D.	1.522	+5	14.0	9.0	−35.7†	12 days after thyroidectomy.
Mrs A. C.	1.490	+49	13.7	21.9	+60.0	Patient responded to treatment.
			13.7	19.0	+38.7	
	1.520	+35	14.0	16.4	+17.1	
	1.570	+23	14.0	20.0	+42.9	
			14.3	16.2	+13.3	
			14.3	15.2	+6.3	
Mr. I. L.	1.771	+27	19.5	26.0	+33.3	6 days after thyroidectomy.
			19.5	21.7	+11.3	
	1.824	+27	20.0	19.6	−2.0	
	1.823	+7	20.0	21.1	+5.5	

						Remarks
Mrs. S. D.	1.303	+50	11.7	.17.0	+45.2	Postoperative.
			11.8	16.6	+40.6	
	1.315	+25	11.8	12.5	+6.0	
Mr. J. W.	1.770	+56	19.5	25.7	+31.8	15 days after thyroidectomy.
	1.792	+56	19.8	29.0	+46.5	
	1.815	+58	19.9	28.0	+40.7	
	1.816	+61(?)	19.9	25.3	+27.1	
			19.9	32.0	+60.8	
			20.2	21.0	+ 4.0	
	1.837	+25	20.2	23.7	+17.3	
Mrs. E. B.	1.446	+71	13.0	16.9	+31.1	Graves' disease complicated by bronchopneumonia; patient died.
	1.415	+36	12.9	16.0	+25.0	
	1.392	+42	12.8	19.6	+53.1	
			12.8	22.3(?)	+74.2	
Miss E. G.	1.468	+54	13.4	21.9	+63.4	
	1.451	+54	13.2	15.3	+16.0	
	1.475	+21	13.4	9.1	−32.1†	
Leucemia, Mr. G.	1.595	+33	17.5	34.1	+94.8	Patient practically *in extremis*.
			17.5	32.5	+85.7	
			17.5	26.1	+49.1	Marked clinical improvement.
			17.5	23.3	+33.1	

TABLE I—*Concluded.*

Pathological condition.	Surface area.*	Basal metabolism.	Pigment output per day.			Remarks.
			Calculated normal value.	Actual value.	Deviation from normal.	
	sq.m.	*per cent*	*units*	*units* † §	*per cent*	
Lobar pneumonia, Mr. F. C.	1.429		15.8	25.2 21.1	+33.5	} During pyrexia.
			15.8	49.7 33.4	+111.4	
			15.8	39.6 29.2	+84.8	} In convalescence.
			15.8	13.1 13.0	−17.7	
Mr. T. R.	1.767		19.5	24.6 24.3	+24.6	} During pyrexia.
			19.5	40.4 32.8	+68.2	
			19.5	15.1 15.1	−22.5	} Patient convalescing.
			19.5	14.5 14.3	−26.7	
Mr. C. C.	1.671		18.5	23.7 23.7	+28.1	} During pyrexia.
			18.5	53.3 39.4	+113.0	
			18.5	25.3 20.3	+9.7	
			18.5	17.8 16.8	−9.2	} During convalescence.
			18.5	30.2 24.0	+29.7	
			18.5	14.5 14.4	−22.2	
			18.5	12.4 12.4	−33.0	

* Surface area determined from height and weight data by the Du Bois chart in the thyroid and leucemia patients and by the Meeh-Rubner formula, surface $= 11.3 \times W^{\frac{2}{3}}$, in the pneumonia cases.

† Specimen probably incomplete.

‡ Total pigment output.

§ Urochrome output; extraneous pigments, urobilin and hematoporphyrin, removed.

particularly, to correlate the clinical progress, basal metabolism, and pigment output.

Methods.

The output of the normal urinary pigment was determined colorimetrically, by use of the alizarin-aniline orange standard (1), upon 24 hour urine specimens. In all instances the urine specimens were collected upon consecutive days, in some cases over hospitalization periods 2 months long. To check the reliability of collections the creatinine content of the urines was determined from time to time by Folin's (2) microchemical procedure. Pigment determinations were made, as a rule, weekly, the collected urines being preserved under toluene and kept in the ice box. Pigment and metabolism determinations were made either on the same day or within a few days of each other.

As has been pointed out in the first paper (1), in a study of the urochrome output in hospitalized individuals it is necessary to eliminate the presence in the urine of extraneous and pathological coloring matters. The administration of certain medicinal agents —rhubarb, as an example—was therefore avoided. The possibility, also, that increments in urinary color may have been due to the excretion of abnormal quantities of urobilin and hematoporphyrin rather than to urochrome *per se* was eliminated. This was done by carrying out control pigment determinations upon urines from which the pathological pigments had been largely removed by a modification of Garrod's technique (1).

In order to interpret the clinical data correctly it was essential to have some idea of the quantity of pigment that pathological individuals would excrete if they were normal. In the first paper (1), data showing the direct proportionality of pigment output and surface area in twenty-three normal subjects (including three different species) have been presented. In plotting these data of the pigment output per day against the surface area it is obvious that a majority of the points fall along a straight line. The slant of this line indicates that the pigment output is approximately 11.0 units per square meter of body surface. In the case of man, normal distribution curves (1) brought out the fact that the pigment output of females was 17 per cent lower than that of males. Upon the basis of these findings two formulæ were used to calculate

the normal pigment value.   Pigment output of male subjects = 11.0 × surface area.   Pigment output of female patients = 11.0 × 0.83 × surface area.

Results.

The output of the urinary pigment was found to be above normal in all the individuals with exophthalmic goiter.   In these cases there was a distinct parallelism of the per cent increase in urochrome elimination and in basal metabolic rate.   In several patients upon whom thyroidectomy had been performed, the urinary pigment value fell to normal or, indeed, somewhat below the calculated normal level after the operation.   In a patient (Mrs. E. K.) who proved resistant to therapy both pigment output and basal metabolism persisted at levels far above normal. In another individual (Mrs. A. C.), in whom response to treatment was clinically apparent and whose basal metabolic rate gradually fell towards normal, the output of pigment also fell.

In the leucemia case the relationship of pigment output and basal metabolism was similar to that observed in the thyroid patients.

During the pyrexic stage of lobar pneumonia the urochrome output was above normal, while during convalescence from this disease the output of pigment was consistently below the normal level.   The pathological pigments, both urobilin and hematoporphyrin (identified by chemical tests and spectroscopically), were found in appreciable quantities only in the pneumonia urines.   A summary of the essential findings is given in Table I.

The conclusion (1), reached mainly from experimental evidence, that the output of the normal urinary pigment is proportional to the intensity of the endogenous metabolism is fortified by the data obtained from the present series of carefully controlled clinical cases.   This is borne out by the abnormally high pigment elimination in pathological conditions associated with an elevated metabolism, by the parallelism of pigment output and basal metabolism, and by the fall of the output of pigment after thyroidectomy in Graves' disease and after the cessation of fever in pneumonia.

SUMMARY AND CONCLUSIONS.

The study of the output of the normal urinary pigment in a new series of clinical cases has furnished further evidence in support of the idea that the quantity of pigment excreted is proportional to the metabolism.

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CONFIGURATIONAL RELATIONSHIPS OF 2-HYDROXY-BUTYRIC AND LACTIC ACIDS.

A Reply.

By GEORGE WILLIAM CLOUGH.

(From the Department of Chemistry, Royal Veterinary College, London, England.)

In a recent paper with the above title Levene and Haller[1] have shown in an elegant manner that the optically active form of 2-hydroxybutyric acid which is dextrorotatory in aqueous solution at ordinary temperature, but which yields levorotatory salts and esters, possesses the same configuration as *d*-lactic acid or *l*-tartaric acid. These authors designate the acid in question dextro-2-hydroxybutyric acid and state that I[2] reached the opposite conclusion with regard to the configuration of this compound, this conclusion being based on an error, inasmuch as the direction of the rotation of the free acid was not taken into consideration. In order to distinguish the enantiomorphous forms of a compound from one another, the conventional symbols *d* and *l* have usually been assigned to them in a somewhat arbitrary manner. Thus, *d*-lactic acid is the form which is dextrorotatory in aqueous solution at ordinary temperature but which gives rise to levorotatory salts and esters, while *l*-aspartic acid, which may be prepared from natural *l*-asparagine, is dextrorotatory in aqueous solution. Guye and Jordan,[3] who resolved 2-hydroxybutyric acid into its optically active forms, termed that variety of this acid, the salts and esters of which were levorotatory, "l'acide α-oxybutyrique gauche." Beilstein[4] refers to the same

[1] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1927, lxxiv, 343.

[2] Clough, G. W., *J. Chem. Soc.*, 1918, cxiii, 533.

[3] Guye, P. A., and Jordan, C., *Bull. Soc. chim.*, 1896, xv, series 3, 474.

[4] Beilstein, S. K., Handbuch der organischen Chemie, Berlin, 4th edition, 1921, iii, 301.

compound as "*l*-α-Oxy-buttersäure." The present author also designated this compound *l*-α-hydroxybutyric acid and expressed the view that it was configurationally related to *l*-tartaric acid. It is thus evident that I made no error concerning either the conventional or the configurational designations of the 2-hydroxybutyric acids, but that my conclusion regarding the configurations of the optically active compound under consideration is in accordance with the result now obtained by Levene and Haller.

OBSERVATIONS ON THE NATURE OF THE SUGAR OF NORMAL URINE.

By ISIDOR GREENWALD, JOSEPH GROSS, AND GRACE McGUIRE.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

In 1922, Folin and Berglund (1) stated that the sugars of normal urine had their origin largely in (1) "foreign, partly or wholly unusable, carbohydrate materials present in grains, vegetables, and fruits," and (2) "decomposition products due to the heat used in cooking, canning, and baking" of such food. Their evidence for the latter part of this statement was, however, only slight and was criticized by Benedict and Osterberg (2).

In a previous communication from this laboratory (3), it was shown that the excretion of sugar in the urine of a dog was greater when toasted cracker meal was fed than when ordinary cracker meal was used. It was also found, in both man and dog, that the feeding of a well toasted food (Grape-Nuts) resulted in a marked increase in the excretion of sugars. This could not have been due to the maltose contained in the food for the administration of a large amount of maltose produced no such increase.

Using a pure yeast and the Haldane differential manometer, Lund and Wolf (4) obtained no evidence of gas formation in normal urine, although 0.05 per cent of added glucose gave a difference in manometer readings of 30 mm. Patterson (5) found no change in the reducing action of urine after treatment with yeast. Some years ago, Mathews (6) called attention to the fact that yeast would remove small quantities of glucose from solution within a few minutes and Eagle (7) has recently made use of this fact in a method designed to detect small amounts of glucose. Although added glucose could always be detected, none could be found in normal urine, in some cases not even after the subjects had ingested considerable quantities of glucose.

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Pucher (8) prepared osazones from several urines but concluded that they were not identical with glucosazone. Patterson (5) used the filtrate from the precipitate obtained by adding mercuric nitrate and sodium carbonate to urine and obtained 0.1 gm. of an osazone from 5 liters of urine. Although analyses for nitrogen, carbon, and hydrogen indicated that it was a hexosazone, the crystal form was not that of phenylglucosazone and even after repeated crystallization from several solvents, it remained more soluble in hot water than is phenylglucosazone.

In unpublished experiments, we obtained from normal human urine, material which was non-fermentable or only slightly fermentable, yet reduced and formed phenylglucosazone without previous hydrolysis with acid. Somewhat similar observations had previously been recorded by Geyer (9) and by Baisch (10).

In view of the results of the feeding experiments already referred to, it seemed to be of interest to examine Grape-Nuts for the presence of such material. As is shown in the experimental part, its presence was demonstrated. Considering the reducing action of the material submitted to the test, the yield of osazone was small but, in view of the well known interfering action of both non-reacting carbohydrates and excess of phenylhydrazine upon the yield of phenylosazones, this is not altogether surprising.

Pure carbohydrates of this kind, viz., non-fermentable but capable of reducing sugar reagents and of yielding phenylglucosazone, have become known only recently. Pictet and his associates have prepared and studied the properties of levulosan (11) and isosaccharosan (12), both of which reduce Fehling's solution and yield phenylglucosazone, though they are not fermented by yeast, without previous hydrolysis.

Neither levulosan nor isosaccharosan was immediately available to us. We determined to use the mixture of these, and other substances, obtained by heating sucrose *in vacuo*, at 155°, until it melted. In 40 gm. lots, this generally required from 3 to 4 hours. After cooling, the material was like taffy. In 20 per cent solution, $[\alpha]_D$ was about 40°. After fermentation with yeast until no more gas was produced, although added sucrose was promptly attacked, the solution was still slightly dextrorotatory. Treatment with alcohol yielded a precipitate which, when dried *in vacuo*, weighed about 10 per cent as much as the sucrose originally used. The

reducing action towards the Benedict-Osterberg sodium picrate-carbonate reagent (13) was about 20 per cent of that of the same amount of glucose while with Benedict's copper reagent (14), the relative reducing action was only about 16 per cent. After hydrolysis with acids, the reducing action by either method was about 75 per cent of that of glucose. Both the raw material and the alcohol precipitate yielded phenylglucosazone in large quantity

TABLE I.

Effect of Ingestion of Heated Sucrose upon Excretion of Sugar by a Dog.

Dog 60. Female; weight 11 kilos. The daily diet consisted of 40 gm. of cracker meal, 40 gm. of dried meat residue, 35 cc. of maize oil, 12 gm. of bone ash, and 400 cc. of water.

Date.	Nitrogen.	Urine.				Feces.		
		Sugar.				Amount.	Sugar per gm., picrate-hydroxide method.	
		Benedict's copper method.	Sumner's method.	Picrate-hydroxide method.			Before hydrolysis.	After hydrolysis.
				Before hydrolysis.	After hydrolysis.			
Feb.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*
21	7.23	0.132	0.120	0.167	0.256	20.0	4.4	4.5
22	7.07	0.154	0.153	0.186	0.282	17.5	4.0	4.3
23	6.21	0.264	0.279	0.279	0.705	16.0	1.4	3.7
24	7.28	0.166	0.147	0.169	0.267	28.1	6.9	12.9
25	7.37	0.155	0.125	0.183	0.218	13.8	4.3	7.4
26	6.94	0.148	0.119	0.190	0.212	28.6	3.1	4.0
27	6.99	0.142	0.131	0.181	0.195	18.0	3.8	5.2

On Feb. 23, added to the food the taffy obtained by heating 40 gm. of sucrose, *in vacuo*, at 155° until it melted (4 hrs.).

when treated in the usual manner with phenylhydrazine hydrochloride and sodium acetate.

After feeding heated sucrose to one dog (Table I), there was a decided increase in the sugar content of the urine. This was even more marked in determinations made on urine filtrates that had been heated with hydrochloric acid, indicating the presence of a di- or polysaccharide. That all the sugar fed was not absorbed is shown by the increase in the sugar content of the feces voided on the following day.

Another dog (Table II) received, at different times, both heated sucrose and the alcohol-precipitated non-fermentable material

TABLE II.

Effect of Ingestion of Heated Sucrose or of Alcohol-Precipitated Non-Fermentable Portion Thereof on Excretion of Sugar by a Dog.

Dog 59.   Female; weight 7.5 kilos.   The daily diet consisted of 10 gm. of dried meat residue, 10 gm. of cracker meal, 10 cc. of maize oil, 8 gm. of bone ash, 10 gm. of sucrose, and 150 cc. of water.

Date.	Nitrogen.	Urine.				Feces.		
		Sugar.				Amount.	Sugar per gm., picrate-hydroxide method.	
		Benedict's copper method.	Sumner's method.	Picrate-hydroxide method.				
				Before hydrolysis.	After hydrolysis.		Before hydrolysis.	After hydrolysis.
Feb.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*
7	2.35	0.146	0.077	0.132	0.197			
8	2.23	0.091	0.102	0.140	0.145	23.5	2.5	3.5
9	1.79	0.070	0.055	0.096	0.121			
10	2.06	0.047	0.052	0.109	0.125	24.7	2.9	2.7
11	2.35	0.097	0.069	0.116	0.202			
12	2.56	0.074	0.061	0.091	0.130			
13	2.28	0.080	0.050	0.091	0.138	19.9	4.8	10.5
14	1.71	0.068	0.060	0.078	0.109			
15	2.54	0.095	0.109	0.135	0.170			
16	2.43	0.110	0.083	0.129	0.191			
17	2.08	0.084	0.072	0.091	0.170	26.8	2.5	3.1
18	2.54	0.161	0.128	0.143	0.357	21.3	2.3	3.1
19	2.68	0.084	0.084	0.126	0.106	13.8	3.0	4.1
20	1.10	0.047	0.038	0.057	0.077			
21	0.65	0.018	0.016	0.023	0.034	19.3	4.0	5.2

On Feb. 11, added 2.87 gm. of the dried material precipitated by alcohol from the non-fermentable portion of heated sucrose.   On Feb. 18, substituted 10 gm. of sucrose previously heated for 3 hrs. at 155°, *in vacuo*, for the pure sucrose of the usual diet.

obtained therefrom.   The heated sucrose had the same effect as it had in the previous experiment but the alcohol-precipitated material increased the excretion of sugar in the urine but very

little.   The increase in the sugar content of the feces was very marked and accounted for at least half of the material fed.

When 40 gm. of heated sucrose were fed to a man (Table III), there was no increase in the excretion of sugar in the urine.   In this experiment, Eagle's fermentation technique (7) was also used. Not the slightest amount of fermentable sugar was detected.

TABLE III.

Effect of Ingestion of 40 Gm. of Sucrose Previously Heated to 155°, in Vacuo, for 3 Hours, upon Excretion of Nitrogen and Sugars.

Subject I. G.   Male; age 39 years, weight 71 kilos.

Time.	Excretion per hour.						
	Volume.	Nitrogen.	Sugar.				
			Benedict-Osterberg, picrate-hydroxide method.	Sumner's method.	Eagle's fermentation technique.		
					Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12–7	82	0.73	0.038	0.027	0.024	0.026	−0.002
7–9	64	0.55	0.029	0.021	0.029	0.021	0.008
9–11	45	0.45	0.020	0.017	0.020	0.016	0.004
11–1	88	0.50	0.028	0.023	0.023	0.023	0.000
1–3	117	0.56	0.036	0.026	0.026	0.027	−0.001
7–9	59	0.42	0.048	0.047	0.036	0.035	0.001
9–11	50	0.46	0.047	0.042	0.035	0.033	0.002
11–1	85	0.51	0.043	0.044	0.035	0.037	−0.002
1–3	86	0.34	0.037	0.028	0.022	0.024	−0.002
4–5	63	0.41	0.033	0.030	0.026	0.027	−0.001
5–7	47	0.40	0.029	0.026	0.024	0.023	0.001

Sugar ingested at 11 a.m. in both experiments.

However, other specimens of urine obtained from this subject at about this time did contain a reducing substance removed by treatment with yeast.   Since this subject had only 3 years earlier shown an apparently perfect tolerance for 100 gm. of glucose, it was determined to test his tolerance again, using Eagle's technique in testing for the presence of fermentable sugar.   Urine collected

in 2 hour periods after the ingestion of 100 gm. of glucose (92 gm. of anhydrous glucose) showed absolutely no sign of the presence of fermentable sugar (Table IV). But there was a decided difference between the reducing actions, before and after treatment with yeast, of the urines obtained after the ingestion of a mixed meal.

The excretion of sugar as determined by any one of three differ-

TABLE IV.

Effect of Ingestion of Glucose and of a Mixed Meal on the Hourly Excretion of Nitrogen and Sugar.

Subject I. G.   Male; age 39 years, weight 71 kilos.

Time.	Volume.	Nitrogen.	Benedict-Osterberg, picrate-hydroxide method.	Sumner's method.	Eagle's fermentation technique.		
					Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12.30– 6.30	55	0.70	0.056	0.037	0.035	0.033	0.002
6.30– 8.30	77	0.60	0.032	0.030	0.024	0.025	−0.001
8.30–10.30	55	0.55	0.034	0.028	0.024	0.022	0.001
10.30–12.30	38	0.49	0.023	0.020	0.020	0.019	0.001
12.30– 2.30	35	0.47	0.019	0.020	0.019	0.018	0.001
2.30– 4.30	45	0.55	0.030	0.023	0.029	0.026	0.003
4.30– 7.30	38	0.52	0.049	0.039	0.044	0.027	0.017

At 10.30 a.m., 100 gm. of glucose (92 gm. of anhydrous glucose) in 400 cc. of water.   Between 4.45 and 5.30 p.m., dinner of relishes, vegetable soup, ravioli, chicken, lettuce, apple pie, coffee (2 lumps sugar), bread, and butter.

ent methods was not only not increased but was actually diminished after the ingestion of glucose.   There was a fairly close proportion between the amount of sugar excreted and that of nitrogen.   Believing that this same relation might obtain after a protein-fat meal, the excretion of sugar and nitrogen after such a meal was next studied (Table V).   There was no evident relation between the amounts of nitrogen and sugar in the urine.

The appearance of fermentable sugar in the urine after a mixed meal might have been due to lowered tolerance for carbohydrates during the digestion of protein and fat, as was suggested by Benedict, Osterberg, and Neuwirth (15). Accordingly, 100 gm. of glucose were taken with a protein-fat meal of the same kind as was employed in the preceding experiment. Just as in the first experiment of this series, the amount of total sugar in the urine was *diminished* after the ingestion of glucose and eggs, just as was the amount of nitrogen in the urine. Later, both rose to the previous,

TABLE V.

Effect of Ingestion of a Protein-Fat Meal on the Hourly Excretion of Nitrogen and Sugar.

Subject I. G.   Male; age 39 years, weight 71 kilos.

Time.			Excretion per hour.				
	Volume.	Nitrogen.	Sugar.				
			Benedict-Osterberg, picrate-hydroxide method.	Sumner's method.	Eagle's fermentation technique.		
					Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
7–9	66	0.58	0.040	0.029	0.025	0.021	0.004
9–11	40	0.45	0.027	0.018	0.019	0.016	0.003
11–1	18	0.28	0.014	0.006	0.012	0.011	0.001
1–3	42	0.72	0.016	0.012	0.026	0.021	0.005
3–5	43	0.74	0.019	0.012	0.022	0.020	0.002
5–7	30	0.51	0.018	0.007	0.015	0.014	0.001

At 11 a.m., ate 6 hard-boiled eggs, weighing 320 gm., and calculated to contain 42 gm. of protein, 38 gm. of fat, and to furnish 510 calories.

or to slightly higher, levels. In this experiment, the subsequent ingestion of a mixed meal produced only a slight effect, if any, upon the excretion of sugar.

These experiments were repeated upon three other subjects. In only two of them was the excretion of sugar increased at all after the ingestion of glucose. In one of these, the excretion of nitrogen was also increased so that the ratio of sugar to nitrogen in the urine was decreased rather than increased. In every case, there was an increase in the excretion of sugar after a mixed meal.

Examination for fermentable sugars by Eagle's technique showed that the increases after a mixed meal were as great as, or even greater than, those observed in the same subject after the ingestion of 80 gm. of glucose, even when this was accompanied by a meal of four to six hard-boiled eggs.

In his own experiments, Eagle found no fermentable sugar in the urine after a mixed meal. We have found some. It can scarcely have been due to a leakage of glucose through the kidneys, for no larger quantities of such sugar were found after the ingestion of a large amount of glucose, even when this was accompanied by enough fat and protein to constitute a fairly substantial meal, providing, in all, almost one-third of the subject's daily energy requirements.

Eagle has shown that small amounts of glucose are removed by treatment with yeast for 40 minutes. This has been confirmed by us. But the converse is not necessarily true. Our experiments indicate quite definitely that it is not, and that normal urines may contain reducing substances other than glucose that are removed by treatment with yeast for 40 minutes.

That most of the sugar of normal urine is not glucose is recognized by all. In a previous publication (3), attention was called to the rather large amounts of this substance, or substances, excreted when the subject was on a carbohydrate-free diet. It was then suggested that this material might be pentose. Subsequent work has failed to confirm this view. Whatever the substance may be, it yields not pentosazone, but phenylglucosazone, identified as such by its melting point, rotation, and nitrogen content (16).

There is one possibility that does not seem to have been considered. The sugar may be of bacterial origin. Dochez and Avery (17) found a soluble specific substance precipitable by antipneumococcus serum in the urine of patients with pneumonia and Avery and Heidelberger (18) demonstrated that the specific substances obtained from cultures of pneumococci are all sugar derivatives, resisting digestive enzymes but yielding glucose and other substances, on hydrolysis with acids. That other organisms may form similar compounds has been shown by Heidelberger, Goebel, and Avery (19), by Laidlaw and Dudley (20), by Mueller (21), and by Furth and Landsteiner (22). It seems quite con-

ceivable, therefore, that the bacteria normally present in the intestinal tract should form such carbohydrates and that these, with or without previous alteration by digestive enzymes, should be absorbed and then excreted in the urine.

Direct evidence of the presence of bacterial carbohydrate in normal urine has not yet been obtained, nor, so far as we are aware, has it been sought. We hope to investigate this question in the near future.

EXPERIMENTAL.

Isolation of an Osazone from the Non-Fermentable Portion of Grape-Nuts.—340 gm. of Grape-Nuts were mixed with 5000 cc. of water and two cakes of compressed yeast and kept at 37° for 24 hours. After filtering, the material was evaporated under diminished pressure, with a bath temperature of less than 40°, to about 2000 cc. A sample was tested with yeast. No gas was formed on standing overnight. Another sample to which 1 per cent of glucose had been added gave a large amount of gas within 20 minutes. Analyses of the liquid before and after heating 1 cc. with 30 cc. of water and 6 cc. of 0.67 N HCl for 3 hours at 100° gave the following results, in terms of gm. per liter.

Method	Sodium carbon- ate- picrate.	Sodium picrate- hydrox- ide.	Sumner.	Folin- Wu.	Folin- Svedberg.
Before hydrolysis	10.96	9.91	11.05	3.75	2.76
After hydrolysis	33.95	31.48	33.30	26.56	21.68

2 liters of this solution (before hydrolysis) were evaporated under diminished pressure to about 100 cc. Tests with yeast were repeated. Again no gas was formed although added glucose was rapidly fermented.

To the remainder, 30 gm. of phenylhydrazine hydrochloride and 50 gm. of crystallized sodium acetate were added. The mixture was heated for 2 hours in a boiling water bath. After it had been allowed to cool and to stand at room temperature overnight, an unsuccessful attempt was made to filter the mixture. The precipitate was separated by centrifuging and washed with water in the same manner. It was then dried in a vacuum desiccator

over sulfuric acid and extracted with boiling chloroform. After recrystallizing from 20 per cent alcohol, it was again dried and extracted with hot chloroform. After again recrystallizing from 20 per cent alcohol, it melted at 203° and contained 15.3 per cent nitrogen. 7.088 mg. yielded 0.942 cc. of N_2 at 24° and 759.3 mm. Found, 15.3 per cent N_2. Calculated, 15.6 per cent.

The total yield of purified osazone was about 0.20 gm. When it was dissolved in the usual alcohol-pyridine mixture, the color of the solution was too dark to permit of polarimetric readings.

Metabolism Experiments.—The methods of analysis used were the following. Benedict and Osterberg's sodium-picrate-hydroxide (23), Benedict's copper method (14), the same after fementation, according to Eagle (7), and Sumner's method (24). For the determination of the increase in reducing action after hydrolysis, the filtrates obtained after treating the urine with animal charcoal, as in the sodium-picrate-hydroxide method were treated with $\frac{1}{5}$ volume of 0.67 N HCl in a boiling water bath for 3 hours. At the end of this time, the liquids were generally no longer acid to Congo red paper. If they were, they were neutralized to Congo paper but kept acid to litmus. After diluting to a definite volume, they were again shaken with charcoal.

The feces were first dried at 100°, then allowed to stand in the laboratory for 2 or 3 days, weighed, ground, and washed into a 200 cc. flask with about 175 cc. of water. After standing overnight, a little lead acetate was added, the mixture was diluted to the mark, and well shaken. It was then filtered, the filtrate was freed of excess of lead with H_2S, and the filtrate from the lead sulfide was then aerated. The liquid was then treated exactly as were the urines.

In the experiments upon dogs, the animals were fed at 9.30 a.m. each day. The urines were collected without catheterization.

The human subjects took their last meal before the experiment at 6 or 7 p.m. The bladder was emptied at about midnight. The night urine and that for one or two 2 hour fasting periods were then collected. After the ingestion of the test material, the urines were collected for three or four 2 hour periods. The subjects then ate a meal of their own selection and continued the collection of urine for two additional periods.

In order to facilitate comparison, all the data for the human experi-

TABLE VI.

Effect of Ingestion of Glucose with a Protein-Fat Meal, and Also of a Subsequent Mixed Meal upon the Hourly Excretion of Nitrogen and Sugar in the Urine.

Subject I. G.   Male; age 39 years, weight 71 kilos.

Time.	Volume.	Nitrogen.	Benedict-Osterberg, picrate-hydroxide method.	Sumner's method.	Eagle's fermentation technique.		
					Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
6–8	44	0.63	0.035	0.014	0.029	0.024	0.005
8–10	113	0.63	0.037	0.026	0.024	0.024	0.000
10–12	34	0.36	0.019	0.010	0.014	0.013	0.001
12–2	40	0.50	0.022	0.013	0.019	0.017	0.002
2–4	65	0.77	0.030	0.025	0.025	0.023	0.002
4–6	123	0.74	0.028	0.025	0.022	0.022	0.000
6–8	97	0.64	0.026	0.027	0.024	0.023	0.001
8–10	69	0.63	0.034	0.034	0.028	0.029	−0.001
10–6.30	73	0.64	0.033	0.030	0.025	0.027	−0.002

At 10 a.m., 100 gm. of glucose (92 gm. of anhydrous glucose) and 6 hard-boiled eggs, weighing 320 gm. (calculated to contain 42 gm. of protein, 38 gm. of fat, and to furnish 510 calories).   Between 6.10 and 6.50 p.m., dinner of clam chowder, baked shad, potato, cauliflower, green peas, lettuce, cucumber, preserved plums, blackberry pie, milk, bread, and butter.

TABLE VII.

Effect of Ingestion of Glucose and of a Subsequent Mixed Meal upon the Excretion of Nitrogen and Sugar in the Urine.

Subject A. B.   Female; age 30 years, weight 48 kilos.

Time.	Excretion per hour.					
	Volume.	Nitrogen.	Sugar.			
			Sumner's method.	Eagle's fermentation technique.		
				Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
11.30–6	38	0.32	0.017	0.017	0.014	0.003
6–8	115	0.27	0.012	0.010	0.010	0.000
8–10	145	0.36	0.018	0.016	0.014	0.002
10–12	140	0.28	0.018	0.014	0.013	0.001
12–2	37	0.19	0.012	0.009	0.008	0.001
2–4.30	36	0.25	0.023	0.019	0.017	0.002
4.30–6.30	17	0.23	0.014	0.018	0.016	0.002

At 8 a.m., 80 gm. of glucose (74 gm. of anhydrous glucose) in water, with juice of 1 lemon.   At 2 p.m., juice of 1 orange, 1 egg, 1 tomato, 2 small slices of bread and butter, 1 cup of coffee with cream, and 3 small dominos of sugar.

TABLE VIII.

*Effect of Ingestion of Glucose and of a Subsequent Mixed Meal upon
the Hourly Excretion of Nitrogen and Sugar.*

Subject J. G.   Male; age 32 years, weight 66 kilos.

Time.	Excretion per hour.					
	Volume.	Nitrogen.	Sugar.			
			Sumner's method.	Eagle's fermentation technique.		
				Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12–6	18	0.25	0.017	0.017	0.019	−0.002
6–8	23	0.18	0.010	0.013	0.012	0.001
8–10	22	0.25	0.010	0.013	0.012	0.001
10–12	26	0.43	0.018	0.018	0.015	0.003
12–2	34	0.34	0.015	0.013	0.008	0.005
2–4	26	0.29	0.015	0.013	0.011	0.002
4–6	30	0.50	0.027	0.030	0.024	0.006
6–8	22	0.38	0.025	0.026	0.021	0.005

At 10 a.m., 80 gm. of glucose (74 gm. of anhydrous glucose) in water.
At 4 p.m., dinner of 2 hard-boiled eggs, ½ pint of strawberries, 200 cc. of
sour cream, lettuce, 4 slices of rye bread, butter, 1 bun, 1 cup of cocoa with
3 teaspoonfuls of sugar.

TABLE IX.

Effect of Ingestion of Glucose with a Protein-Fat Meal and Also of a Subsequent Mixed Meal upon the Hourly Excretion of Nitrogen and Sugar in the Urine.

Subject J. G.   Male; age 32 years, weight 66 kilos.

Time.	Excretion per hour.					
	Volume.	Nitrogen.	Sugar.			
			Sumner's method.	Eagle's fermentation technique.		
				Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12–6	15	0.28	0.007	0.023	0.023	0.000
6–8	17	0.30	0.012	0.016	0.014	0.002
8–10	36	0.38	0.016	0.016	0.014	0.002
10–12	136	0.43	0.016	0.015	0.014	0.001
12–2	55	0.54	0.020	0.018	0.015	0.003
2–4	49	0.44	0.018	0.015	0.014	0.001
4–6	28	0.33	0.017	0.018	0.015	0.003
6–8	30	0.42	0.030	0.030	0.026	0.004

At 10 a.m., 80 gm. of glucose (74 gm. of anhydrous glucose) and 276 gm. of hard-boiled eggs (calculated to contain 36 gm. of protein and 33 gm. of fat and to furnish, with the glucose, 737 calories). At 4 p.m., dinner of spaghetti, canned corn, green peas, fried potatoes, 2 buttered rolls, 1 cup of coffee with 2 teaspoonfuls of sugar, and 56 gm. of chocolate.

TABLE X.

Effect of Ingestion of Glucose and of a Subsequent Mixed Meal upon Excretion of Nitrogen and Sugar in the Urine.

Subject I. L.   Male; age 20 years, weight 52 kilos.

Excretion per hour.

Time.	Volume.	Nitrogen.	Sugar.			
			Sumner's method.	Eagle's fermentation technique.		
				Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12–6	23	0.35	0.014	0.018	0.015	0.003
6–8	22	0.31	0.011	0.012	0.010	0.002
8–10	Lost.					
10–12	30	0.35	0.013	0.013	0.011	0.002
12–2	31	0.32	0.013	0.013	0.009	0.004
2–4	82	0.38	0.016	0.015	0.011	0.004
4–6	32	0.40	0.018	0.021	0.017	0.004
6–8	27	0.41	0.018	0.023	0.018	0.005

At 10 a.m., 80 gm. of glucose (74 gm. of anhydrous glucose) in water. At 4 p.m., salad of herring, tomato, lettuce, and cucumber, tea, bread, and butter.

TABLE XI.

Effect of Ingestion of a Glucose with a Protein-Fat Meal and of a Subsequent Mixed Meal upon the Hourly Excretion of Glucose and Nitrogen in the Urine.

Subject I. L.   Male; age 20 years, weight 52 kilos.

Time.	Excretion per hour.					
	Volume.	Nitrogen.	Sugar.			
			Sumner's method.	Eagle's fermentation technique.		
				Before.	After.	Loss.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
12–6	56	0.34	0.015	0.014	0.012	0.002
6–8	19	0.22	0.009	0.009	0.007	0.002
8–10	34	0.30	0.013	0.012	0.009	0.003
10–12	24	0.26	0.013	0.012	0.010	0.002
12–2	26	0.30	0.011	0.012	0.011	0.000
2–4	26	0.35	0.009	0.011	0.011	0.000
4–6	25	0.40	0.010	0.018	0.014	0.004
6–8	20	0.37	0.011	0.018	0.018	0.000

At 10 a.m., 80 gm. of glucose (74 gm. of anhydrous glucose) in water and 196 gm. of hard-boiled eggs, (calculated to contain 26 gm. of protein, 21 gm. of fat, and to yield, with the glucose, 589 calories).   At 4 p.m., pea soup, salad of lettuce, tomato, cucumber, radishes, and scallions, and chocolate pudding.

ments have been computed to an hourly basis. The details of the individual experiments are given in Tables III to XI and seem to require no further description.

SUMMARY AND CONCLUSIONS.

1. The sugar content of the urine may be increased by the ingestion of heated sugars but the increases observed after the ingestion of considerable quantities of such materials seem to be too small to permit us to consider carbohydrates altered in cooking as the source of any considerable part of.the sugar of the urine.

2. Feeding experiments with glucose showed either no increases in the amount of sugar in the urine or increases that were no greater than those observed after a mixed meal. There was no evidence of a lowered tolerance for glucose taken with a protein-fat meal.

3. Some urines seem to contain a reducing substance that is removed by yeast but which is probably not glucose.

4. A possible source of the urinary sugar is suggested.

Addendum.—After this paper had been submitted for publication, Tomcsik's description (25) of a carbohydrate-like specific substance obtained from *Bacillus lactis aerogenes* and *Bacillus coli communis* appeared. Tomcsik refers to the previous isolation, by Emmerling (26) and by Schardinger (27), of a polysaccharide from filtered cultures of bacilli of the *Bacillus lactis aerogenes* group. The materials obtained by Emmerling, Schardinger, and Tomcsik differ from the urinary carbohydrate in that they do not, without previous hydrolysis, reduce alkaline copper solutions.

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THE INFLUENCE OF PROTEINS ON THE SOLUBILITY
OF CALCIUM PHOSPHATE.

By JOSEPH CSAPO.

(*From the Department of Pediatrics, Harvard Medical School, and the Medical
Division of the Children's Hospital, Boston.*)

The difficulty of preparing a physiologically correct solution of
blood plasma salts is generally known. A slight increase of pH
above 7.4 causes precipitation of calcium phosphate and resulting
turbidity, whereas, in the case of blood serum of the same inor-
ganic composition, the pH may vary fairly widely without precipi-
tation occurring. The precipitation of calcium phosphates and
carbonates is an almost regular feature of tissue necrosis. Licht-
witz (1) especially has emphasized the relationship of the destruc-
tion of protein in tissues to their calcification. Shipley, Kramer,
and Howland (2) in their recent *in vitro* studies of the factors in-
fluencing the calcification of rachitic rat cartilage found that cal-
cification was greatly delayed in the presence of 2 per cent of egg
albumin. These various facts indicate that proteins tend to keep
calcium salts in solution or else in suspension. In other words,
the proteins inhibit a deposition of calcium salts which other physi-
cal factors tend to produce.

It is desired to emphasize here that there is a great difference
between the extent to which calcium phosphate can be dissolved
in blood serum and the extent to which serum may hold it in
solution. Recently Holt, La Mer, and Chown (3) have stated
that blood serum is supersaturated with tricalcium phosphate.
They found it impossible to dissolve added tricalcium phosphate
even by extremely prolonged (9 days) shaking. Indeed, during
the shaking, the calcium of the serum actually fell to a lower level,
indicating apparently an initial supersaturation. In this study
the problem was approached from another direction. The plan
used was to prepare mixtures of a solution of monocalcium phos-

phate and blood serum.[1]　A range of pH was obtained by additions of 0.1 N NaOH to these mixtures.　The pH of the mixtures was determined electrometrically at once and they were placed in an ice chest for 16 hours; then, after sharp centrifugation, calcium and phosphorus were measured in the supernatant fluid.　The product of these measurements, expressed as millimols, was taken as an index of the extent to which calcium phosphate was present in solution and suspension.
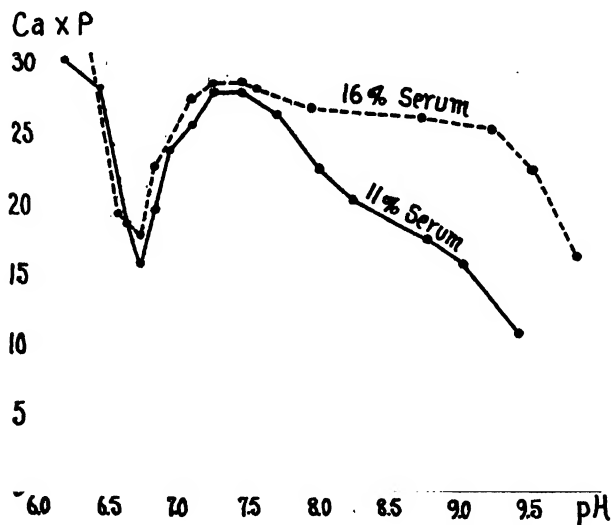
FIG. 1. Effect of pH on Ca×P in a medium containing 11 per cent serum.

The results obtained in this way of a study of the relationship of calcium phosphate solubility in the presence of protein at two levels of concentration are shown by the curves in Fig. 1.　Here may be seen a curious change in the Ca × P values with increase of pH.　There is at first a rapid decline of both curves reaching a minimum at pH 6.7, then a sharp rise which reaches its maximum at about pH 7.3.　Corresponding with these features of the

[1] 20 cc. of a 0.5 per cent solution of monocalcium phosphate were placed in each of a series of 100 cc. volumetric flasks.　After adding serum to provide 11 or 16 per cent, and differing amounts of 0.1 N NaOH, the flasks were filled to the mark with distilled water and the contents thoroughly mixed.

curves a significant change in the appearance of the supernatant fluid takes place. Until pH 6.7 it is entirely clear, but from this point onward with increasing alkalinity it is turbid even after sharp centrifuging. Following the maximal point of rise both curves decline. The curve for the 16 per cent serum series, however, as compared with that for the 11 per cent serum series falls only slightly until an alkalinity of about pH 9.0 is reached. There is thus clearly evident up to this point a greater stabilization of the calcium phosphate suspension in the series containing the higher concentration of protein. It may be mentioned here that in further experiments it was found that the stability of the calcium phosphate suspension may also be improved by reduction of phosphate ion in proportion to calcium in the mixtures. Also, as will presently be shown, the minimal and maximal points in these curves are altered by change in the ratio of protein and phosphate in the mixtures. Moreover, a slight difference in the position of these points was found for different lots of horse serum, possibly due to differences in the total protein concentration or in the albumin-globulin ratio.

An explanation of the above described changes in the Ca $\times$ P values with increasing alkalinity is difficult. Besides the di- or tricalcium phosphate, there are probably also present calcium- and phosphoproteinates in small amounts but varying with pH. Accurate measurement of the various individual factors is not possible. Taking into account the results of the experiments of Holt, La Mer, and Chown, with protein-free solutions, a plausible but only a hypothetical explanation of the observed changes in the Ca $\times$ P values with increasing pH is the following. To begin with the initial decline in the curves; with increasing alkalinity monocalcium phosphate is converted into dicalcium phosphate and to such extent as this latter substance cannot be held in solution by the presence of protein it precipitates. At pH 6.7 tricalcium phosphate begins to be formed and this in the presence of proteins tends to remain in suspension. With further rise in pH this factor increases and, inversely, loss of calcium and phosphorus from the mixtures by formation and precipitation of dicalcium phosphate declines. The curve, therefore, rises until the maximal possible suspension of tricalcium phosphate is obtained. From this point onward the curves demonstrate that hydroxyl ion

concentration and protein exert antagonistic effects on the suspension of tricalcium phosphate so that with increasing alkalinity the size of the suspension depends on the protein concentration present.   Besides tricalcium phosphate in suspension a much smaller and declining value for dicalcium phosphate in solution is probably a factor in the Ca × P value throughout.

The turbidity of the mixtures at the apex of the Ca × P curve, which is near the pH of blood plasma, was so striking a feature in the series as to suggest the question of the extent, if any, to which the presence of protein produces actual solution of calcium phos-
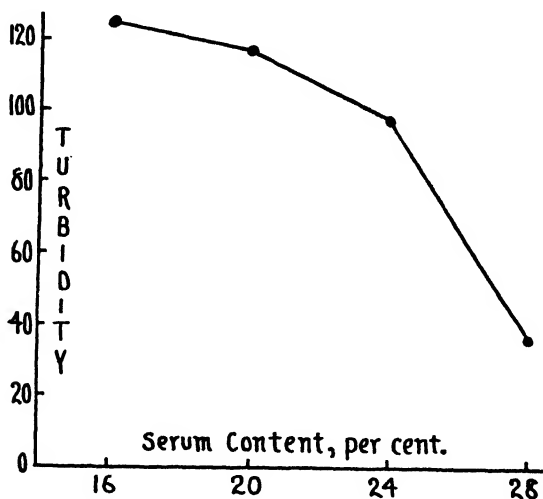
Fig. 2. Effect of increasing concentrations of serum on the turbidity of a serum-monocalcium-phosphate-sodium hydroxide mixture.

phate at this point of reaction.   That dissolved calcium phosphate is present in terms of the protein concentration was shown by the following experiment.   In a series of serum-monocalcium-phosphate-sodium hydroxide mixtures, all of the factors were stationary except serum which was present in steps from 16 to 28 per cent. The pH was 7.25 in all of the mixtures and after centrifuging it was found that the Ca × P value was 36 in all of them.   Turbidity measurements were obtained by means of a Kober nephelometer using as a standard an 0.05 per cent suspension of oleic acid in

hydrochloric acid. Taking the standard suspension as 100, comparative readings were obtained which are plotted in Fig. 2 against the corresponding values for serum content. The curve indicates clearly a reduction of turbidity with increase of serum. Since Ca × P was found stationary, this must mean increasing amounts of calcium phosphate in solution as protein concentration rises. Explanation of this effect of protein cannot be derived from
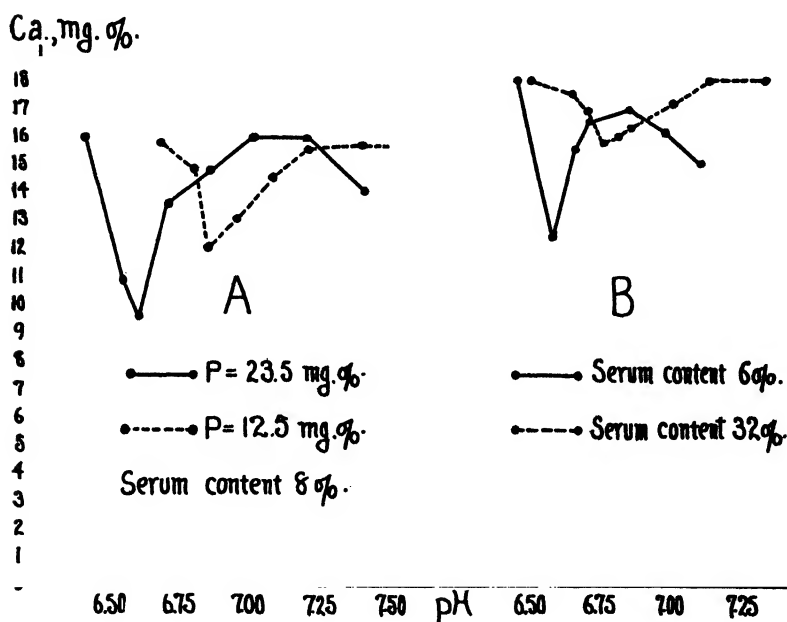
FIG. 3. Effects of phosphate concentration (A) and of serum concentration (B) on the calcium content of serum-monocalcium-phosphate-sodium hydroxide mixtures with special regard to the location of the maximal and minimal values.

these data. Is the increase in the dissolved fraction of the Ca × P value composed of calcium proteinate and dicalcium phosphate or is there also an increased solubility of tricalcium phosphate?

It was mentioned above that the Ca × P value and the location of its maximal and minimal points with reference to pH is a function of the ratio of phosphate and protein present. In Fig. 3

are given curves which demonstrate that the effect produced by increase of protein content may also be obtained by a decrease of phosphate. The data given in Section A (Fig. 3) were obtained from two series of serum-monocalcium-phosphate-sodium hydroxide mixtures, in one of which the initial phosphorus content was 23.5 mg. per cent and in the other 12.5 mg. per cent. In both series the initial calcium content was 16.6 mg. per cent.[2] After standing 16 hours in the ice chest, the mixtures were centrifuged and calcium was determined in the supernatant fluid. The effect of increase of pH is described by the calcium measurements. As may be seen in the curves for the series with the higher phosphate content the minimal point is at pH 6.6 and the maximal at pH 7.0. In the lower phosphate series the points lie at pH 6.85 and pH 7.2 respectively. The data used in constructing the curves in Section B (Fig. 3) are from two series of mixtures identical as regards initial composition except that in one the serum content was 6 per cent and in the other 32 per cent. The curves demonstrate that increase in serum content produces the same effect as does decrease of phosphate; *viz.*, the cardinal points are moved to the right. There is thus evident an antagonism between the effects of the proteins and the phosphates on the relationship between the Ca $\times$ P value and pH. This may be explained in terms of the already indicated hypothesis; *viz.*, low phosphate or high protein concentrations favor the solubility of secondary calcium phosphate and delay the formation of tertiary calcium phosphate.

As a conclusion from these experiments it may be stated that the presence of protein exerts an inhibitory effect on the precipitation of calcium phosphate both by holding it in solution against other physical factors and by supporting it in suspension.

Since the preparation of the above presentation of the results of my experiments, elaborate studies of the solubility of calcium salts in biological fluids have been published by Hastings, Murray, and Sendroy (4) and by Sendroy and Hastings (5). In the third paper of this series, the authors describe a relationship between the solubility of tricalcium phosphate and the serum content of

[2] In the lower phosphate series the calcium content of the initial mixture was obtained by using equal parts of calcium chloride and monocalcium phosphate.

serum-salt solution mixtures. They also state that an apparent calcium supersaturation of serum, following addition of $Ca(HCO_3)_2$ and shaking with $CaCO_3$ and $Ca_3(PO_4)_2$, may be due to suspended calcium salts. The results of my experiments are in good agreement with these findings.

With regard to the influence of pH on the solubility of calcium salts in serum, the evidence of my data differs somewhat from that provided by the experiments of Sendroy and Hastings. They found a continued fall in the Ca content of the serum with increase of pH, the range of pH studied being from 6.2 to 7.5. In my experiments over a similar range of reaction a sharp initial fall of Ca is followed by a rise reaching a maximum between pH 6.8 and 7.5. Differences in procedure doubtless explain this discrepancy in results. Sendroy and Hastings, after adding soluble calcium salts to the serum, rotated it with solid $CaCO_3$ and $Ca_3(PO_4)_2$. In my experiments, $CaCO_3$ was excluded by the addition of an appropriate amount of HCl to the serum, and solid calcium salts were not added to the serum-monocalcium-phosphate-sodium hydroxide mixtures. It would seem likely from the results of Sendroy and Hastings' experiments that the presence of calcium salts in the solid phase tends to produce precipitation from the serum of calcium salts which my data indicate might otherwise have been held in suspension.

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MICRO COLORIMETRIC STUDIES.

I. A MOLYBDIC ACID, STANNOUS CHLORIDE REAGENT. THE MICRO ESTIMATION OF PHOSPHATE AND CALCIUM IN PUS, PLASMA, AND SPINAL FLUID.

By THEODORE KUTTNER* and HARRIET R. COHEN.

(From the Laboratories of the Mount Sinai Hospital, New York.)

During the course of certain biological and pharmacological investigations in this laboratory, it was desirable to have at hand a method for the estimation of small amounts of phosphorus, arsenic, and antimony. As is well known, when these substances in the pentavalent state are acted upon by a reducing agent in an acid mixture in the presence of molybdic acid, a blue solution results. The intensity of color varies proportionately to the amount of phosphorus, arsenic, or antimony present. This reaction has been made the basis of numerous quantitative methods for their estimation, but it is believed that until now the optimum conditions have not been established.

It may be stated at the outset that we were looking for a stable substance which would act as reducing agent and produce the maximum intensity and stability of color in the cold without undue loss of time.

A colorimetric method for phosphorus in which molybdic oxide is used, was published by F. Osmond (1) in 1887, in which the washed, precipitated ammonium phosphomolybdate is reduced with stannous chloride. Taylor and Miller (2), in 1914, used phenylhydrazine to reduce the ammonium phosphomolybdate precipitated from an ashed sample. Hydroquinone was employed by Bell and Doisy (3) in their method for estimating phosphorus in blood. The reaction could be carried out with an excess of molybdate ions and it was found unnecessary to isolate the phosphate as ammonium phosphomolybdate. Briggs (4) modified the method by addition of a little sodium sulfite to the reagent and carried out the reaction in an acid medium, thus obtaining a better blue coloration with greater stability. Denigés (5), at about the same time as Briggs, used 4 "drops" of an ammo-

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* Eugene Meyer, Jr., Fellow.

nium molybdate-sulfuric acid mixture and 2 "drops" of a freshly pre-
pared stannous chloride solution, and waited 10 minutes before comparing
colors.   Stanford and Wheatley (6) have tested Briggs' method and found
it to be accurate and the reaction to be trustworthy for quantitative
estimation of phosphorus, maximum intensity of color being reached in
30 minutes.   Benedict and Theis (7) have introduced a method, whereby
boiling the mixture of molybdic and sulfuric acids with the phosphate com-
pletes the reaction in 15 minutes.   They retained the hydroquinone and
sulfite reducing agent.   Fiske and Subbarow (8) have recommended amino-
naphtholsulfonic acid instead of hydroquinone.   The 1,2,4- and the 1,4,6-
acids are equally effective.   They used sulfite as well as bisulfite of soda and
obtained maximum intensity in 5 minutes in the cold.

Where sulfuric acid is substituted for the sulfite or bisulfite to
prevent oxidation of the hydroquinone, as has been done occasion-
ally, it is less effective and the solution becomes brown in a few
days.   In the present investigation many substances were tested
which act as reducing agents.   Among them were hydroquinone,
diaminophenol, p-aminophenol, monomethyl-p-aminophenol, mon-
ochlorohydroquinone, p-aminosaligenin (edinol), 1, 2, 4- and 1,
6, 8- aminonaphtholsulfonic acids, and stannous chloride.   The
last is by far the best when the concentrations of the reagents used
are properly regulated.   The effect of varying concentrations was
studied as well as certain conditions interfering with the maximum
development of the color.

1.   Molybdic-Sulfuric Acid and Stannous Chloride Reagents.

A.   Use of Sulfuric Acid in Method.—In agreement with Stan-
ford and Wheatley it was found that in the Briggs method,
gradually increasing concentrations of sulfuric acid caused an in-
crease in color until a maximum was reached, then with greater
concentrations of the acid the color intensity decreased.

In the present method the intensity as well as the purity of the
color is regulated by varying the concentration of the reagents.
The optimum acidity of sulfuric acid as shown by Fig. 1 for reduc-
tion of the phosphomolybdic acid lies in a zone between 0.9 and
1.05 N in the final mixture.   Below this optimum acidity, molybdic
acid is itself reduced; the lower the concentration of sulfuric acid,
the greater the reduction of molybdic acid.   An increase of acidity
above the optimum causes at first a retardation in the development
of the color, then with further increase an inhibition of color pro-
duction occurs.   Thus at 1.1 N acidity, it requires about 2 minutes
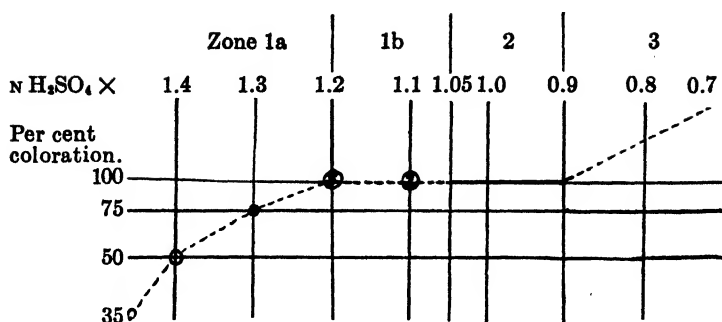
FIG. 1. Effect of increasing concentration of sulfuric acid. Zone 1a = decreased color production of 35, 50, and 75 per cent, also increase of turbidity with increase of acidity. Zone 1b = delayed maximal color production, of 5 minutes at 5, to 2 minutes at 2. Zone 2 = maximal color production in 15 seconds. Zone 3 = production of additional color by reduction of MoO_3.
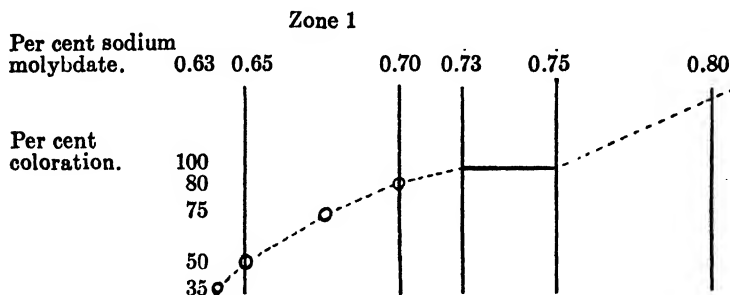
FIG. 2. Effect of increasing concentration of sodium molybdate. Zo: 1 = decreased coloration. Zone 2 = optimum for maximal color. Zone 3 zone of additional color by reduction of MoO_3.
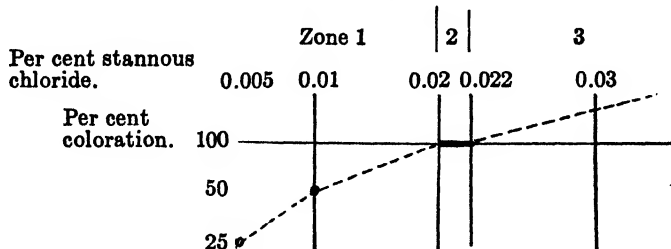
FIG. 3. Effect of increasing concentration of stannous chloride. Zone 1 = decreased coloration. Zone 2 = optimum for maximal color. Zone 3 = additional color by reduction of MoO_3.

to reach maximum intensity, at 1.2 N about 5 minutes, while at 1.3 and 1.4 N the intensity of coloration is lessened to 75 and 50 per cent respectively.   In addition to this retarding effect an increasing turbidity develops as the acidity is increased beyond 1.2 N.

The acidity of the final mixture in our method is about 1 N sulfuric acid; that in the Briggs method is 0.27 N, in the Fiske and Subbarow procedure 0.5 N, in the Benedict and Theis method about 1.9 N, and in that of Roe and Kahn (9) about 1.4 N.

B. *Effect of Increasing Molybdic Acid Concentration.*—A 7.5 per cent solution of sodium molybdate was found convenient for this study.   The optimum concentration of sodium molybdate in the final mixture (as shown in Fig. 2) lies in a zone between 0.73 and 0.75 per cent of sodium molybdate.   Less than this amount effects a decrease in color production, while above this concentration the molybdic acid is reduced in direct proportion to the increased amount.

C. *Stannous Chloride.*—Stannous chloride, though used as a reducing reagent in qualitative colorimetric analysis, has not found favor in quantitative work.   This is perhaps because yellowish brown molybdic oxide complexes are produced simultaneously with the blue color, but in varying amounts, thus resulting in olive-green tints.   This disturbing influence has been eliminated and the use of sulfite found unnecessary.   The optimal concentration of stannous chloride lies in a zone between 0.02 and 0.022 per cent, as shown in Fig. 3.   Stronger solutions reduce molybdic oxide as well as phosphomolybdic acid.

2. Interfering Substances.

Interfering substances can retard color production, inhibit it partly or entirely, or develop a different color in addition.   The degree of interference depends upon the quantity of the disturbing substance.   For example, trichloroacetic acid begins to interfere with maximum color production only at concentrations above 4 per cent in the final mixture.   Hydrochloric acid has a tendency to lessen color stability and retards and inhibits maximum coloration. Thus 2 N hydrochloric acid in the final mixture inhibits color production.   N hydrochloric acid retards and prevents maximum coloration, while 0.5 N hydrochloric acid allows maximum produc-

tion of the color but it soon fades.   Nitric acid interferes above 0.003 per cent, and with a large amount, for instance 1.5 per cent, a green coloration results.   Tartaric acid interferes above 0.002 per cent with inhibition of maximum color, and above 0.04 per cent with reduction of molybdic acid itself.   The effect of citric acid is twice as great.   Hypochlorites interfere strongly.   Up to 0.00004 per cent there is no difference in intensity but a slight difference in the tint, while at 0.00008 per cent a 20 per cent loss of color occurs, and there is a 50 per cent loss of color in the presence of 0.0004 per cent.   Nitrites interfere strongly at concentrations greater than 0.0001 per cent.   Traces of copper or iron salts do not interfere.

Sulfates interfere presumably by depressing the ionization of the sulfuric acid, the interference depending upon the quantity present. Salts of weak acids such as acetates, tartrates, and citrates also interfere, whereas acetic acid itself does not interfere, but causes the development of purplish tints at concentrations above 5 per cent.

Silicates do not interfere at the optimum acidity, up to 4 or 5 times the amount of phosphorus present.

3. General Considerations.

The test is sufficiently sensitive to determine about 0.001 mg. of phosphorus, arsenic, or antimony in the pentavalent state in 5 cc. of solution.   To prevent misleading results, with so sensitive a method, it is obviously necessary to work with pure reagents.

The blue color is produced practically instantaneously, without heating, the reaction being complete in 15 seconds when conditions are right.   The velocity of the reaction is about 5 to 10 times greater than in the Fiske and Subbarow method, about 40 times that of the Denigés procedure, and more than 100 times that of the Briggs or Benedict and Theis methods.   The colors produced by the other methods mentioned have a purplish tint, while the present method yields a better blue with greater intensity.   There is no parallelism between the intensities and velocities of the colors produced by the different methods.   The estimation of the comparative value of the colors is somewhat difficult because of the difference in tint.   In the new method the color is approximately 3 to 4 times greater than that of the Benedict and Theis or

Denigés methods, and about 12 to 15 times that of the Briggs or the Fiske and Subbarow methods.

Based upon the reaction mentioned in this paper, methods for the quantitative determination of phosphorus, arsenic, antimony, and calcium, etc., have been devised. Only methods for phosphates and calcium will be considered in this paper.

4. Description of Methods.

A. Method for Estimation of Phosphates.—The following solutions are necessary and thus far they have remained unchanged after several months.

1. Molybdic-Sulfuric Acid Mixture.—Dissolve 18.75 gm. of sodium molybdate in 2.5 N sulfuric acid; or mix 1 volume of 7.5 per cent sodium molybdate (Kahlbaum's "Zur Analyse") with 1 volume of 10 N sulfuric acid, and add 2 volumes of distilled water. Store in a brown, glass-stoppered bottle.

2. Stannous Chloride Stock Solution.—Dissolve 10 gm. in 25 cc. of concentrated hydrochloric acid. Store in a brown, glass-stoppered bottle. Dilute 0.5 cc. of this stock solution to 100 cc. with distilled water. The diluted reagent does not keep, and should be freshly prepared as needed.

3. Standard Phosphate Stock Solution.—Dissolve 0.4394 gm. of dried monopotassium phosphate in 1 liter of distilled water. Add a few drops of chloroform to prevent mold formation. 1 cc. = 0.1 mg. of phosphorus. Make two standard phosphate solutions by diluting 5 cc. and 10 cc. of the phosphate stock solution in 100 cc. graduated flasks and fill to the mark with water. The solutions will contain respectively 0.005 and 0.01 mg. of phosphorus per cc.

The solution to be tested should contain between 0.4 and 1.2 mg. of phosphorus per 100 cc. (1) Place 2.5 cc. of the sample to be examined in a test-tube graduated at 5 and 10 cc. The sample should contain between 0.01 and 0.03 mg. of phosphorus. (2) Place 2.5 cc. of each standard phosphate solution in two other similarly graduated test-tubes, so that one tube contains 0.0125 mg. and the other 0.025 mg. of phosphorus. (3) Add to each tube 2 cc. of the molybdic-sulfuric acid reagent and mix. (4) Add simultaneously to each of the three tubes 0.5 cc. of the diluted stannous chloride reagent. Insert rubber stoppers and mix without delay by inverting the tubes once or twice.

The color is produced immediately, and although the reaction is complete in 15 seconds if conditions are right, it is advisable to wait a full minute before comparison with the standards is made. Any type colorimeter can be used but it may be necessary to dilute all solutions to 10 cc. if a plunger type is employed. The method of procedure and calculation is carried out in the manner usual for the type of colorimeter employed.

B. Method for Estimation of Calcium.—The estimation of calcium is similar to that of Roe and Kahn (9), the calcium being precipitated as phosphate and the phosphorus estimated colorimetrically. It differs however from theirs by the omission of phenolphthalein and by the use of a standardized alkaline phosphate solution for the precipitation. A phosphate standard expressed in terms of calcium is also used in the present method.

Whether the concentration of alkali present for precipitation has an influence on the character of the precipitate is being investigated, as well as the solubility of calcium phosphate. It appears that under certain conditions the precipitate does not consist of tertiary calcium phosphate $Ca_3(PO_4)_2$ only, but of a mixture containing also dicalcium phosphate $Ca_2H_2(PO_4)_2$. The mixture may vary in the proportion of each substance. The ratio of calcium to phosphorus in the tricalcic phosphate is approximately 4:2, in the dicalcic phosphate 4:3. The latter contains 25 per cent more phosphorus with subsequent greater color production. It is obvious that a precipitate containing a mixture of the two would give erroneous results when compared with a standard prepared with pure calcium salt under different conditions.

The most favorable amount of calcium present for the test to be described is a concentration of about 0.4 to 1.2 mg. per 100 cc. of solution.

The following solutions are necessary:

1. Molybdate-Sulfuric Acid Mixture.

2. Diluted Stannous Chloride Reagent.—Reagents 1 and 2 have been described above under the phosphate method.

3. 10 Per Cent Solution of Sodium Hydroxide Containing 1 Per Cent of Basic Trisodium Phosphate.—Dissolve 1 gm. of the phosphate in 50 cc. of distilled water and mix with 50 cc. of 20 per cent sodium hydroxide (free from silica and calcium). If a precipitate forms it should be allowed to settle for 24 hours or a small portion

may be centrifuged in an hour for immediate use.   In the Roe and Kahn method the phosphate and hydroxide solutions are used separately and may therefore give rise to error in estimating the true calcium value.

4. 50 Per Cent Solution of Alcohol.—Dilute 55 cc. of 95 per cent alcohol with water.   Make faintly alkaline with calcium-free sodium hydroxide, litmus paper being used as indicator.   Make up to 100 cc.

5. Two Standard Phosphate Solutions.—(A) Dilute 51.7 cc. of the phosphate stock solution described in the phosphate method to 1000 cc. in a graduated flask.   2.5 cc. are equivalent to 0.025 mg. of calcium.   (B) Dilute 51.7 cc. of the phosphate stock solution in a graduated flask to 500 cc.   2.5 cc. are equivalent to 0:05 mg. of calcium.

Transfer 5 cc. of the solution of the sample to be analyzed to a centrifuge tube or Pyrex test-tube graduated at 5 and 10 cc.   The solution should have a calcium content of about 0.02 to 0.06 mg. and an acidity of not more than 7 per cent trichloroacetic acid, as previously described.   Add 1 cc. of the alkaline sodium phosphate mixture, mix, set aside for 1 hour, then centrifuge for 3 minutes. Discard the supernatant fluid, catching the last drop on a piece of blotting paper.   Allow to drain 1 to 2 minutes, then wipe the rim of the tube, and wash twice with 5 cc. portions of the faintly alkalinized alcohol, being careful in so doing also to rinse the sides of the tube and to break up the phosphate precipitate.   Drain and wipe the tube after each centrifuging as before.   Add 2 cc. of the molybdic-sulfuric acid mixture to the tube and dissolve the precipitate.   Add 2.5 cc. of water and mix.   If desired 4.5 cc. of a diluted molybdate mixture can be used, prepared by diluting 200 cc. of the molybdate-sulfuric acid mixture with 250 cc. of distilled water.   Into one of two graduated test-tubes transfer 2.5 cc. of calcium Standard A and 2.5 cc. of Standard B into the other; add 2 cc. of molybdic-sulfuric acid mixture to each, and mix. Add 0.5 cc. of the diluted stannous chloride reagent to each standard as well as the unknown and without delay close with a rubber stopper and invert each tube once or twice.   After about 1 minute compare in a colorimeter.   If the plunger type of colorimeter is used proceed in the same manner as described for phosphorus.

Described below is an adaptation of the method on a small

scale, devised for the estimation of phosphates and calcium in 0.1 to 0.2 cc. of blood plasma, spinal fluid, or pus. Calcium may even be determined if only 0.05 cc. of pus is available.

The micro method was primarily devised for calcium determinations on the usually small amounts of pus obtained from the human ear in diseased conditions such as mastoiditis. Friesner and Rosen (10) have shown that pus derived from bone destruction is relatively high in calcium, and their investigation was the stimulus for the development and use of the present micro method.

Pus, unlike plasma, is not a homogeneous fluid and may contain debris from bone or soft tissue. The calcium cannot be directly isolated as oxalate, as Kramer and Tisdall (11) do in the case of blood plasma, even if a sufficient quantity is available for the use of their method. In addition, the calcium isolated as oxalate from the ash of a small sample is too minute in amount to be titrated with potassium permanganate. Less material is required with the Van Slyke and Sendroy (12) gasometric method, in which 1 cc. of blood plasma is sufficient and even less may be taken. Roe and Kahn (9) use 2 cc. of plasma and estimate the phosphorus by the Benedict and Theis (7) method from the precipitated tertiary calcium phosphate. This procedure was tested on ashed samples of small quantities of material. In the use of this method, the exact matching of color depth with the Dubosq colorimeter was prevented because of the somewhat variable greenish tints in the unknowns. The influence of the adventitious yellow is increased and becomes more disturbing as the depth of the liquid increases. This effect is lessened and minimized by the use of a dilution type of colorimeter such as the micro colorimeter (13) described in a previous paper. This instrument also permits employment of smaller samples and less reagent for the test.

Ultimately it was found that phenolphthalein is the disturbing factor, being adsorbed by the calcium precipitate, and the indicator is therefore omitted. Although the greenish tint is entirely eliminated in the new method, the micro colorimeter is retained because of convenience, equal accuracy, and other advantages. The old type of diluting tubes has been replaced by glass-stoppered tubes[1] of uniform bore, graduated to 180 on an etched

[1] The colorimeter and glassware are manufactured by E. Leitz, Inc., 60 East 10th Street, New York.

scale, each division on the scale equalling 0.02 cc.   The use of permanent color standards has been found very convenient, and their preparation will be described in another paper.   A pipette furnished with a rubber nipple, and drawn to a fine bore at the lower end, replaces the old type, permitting the use of smaller drops for dilution or a fine stream for rinsing.   A narrow test-tube (12 × 120 mm.) graduated at 1 and 2 cc. has been found useful for the precipitation of the protein.

Micro Estimation of Phosphates.

Two permanent standards are used: A, equivalent to 0.0025 mg. of phosphorus, and B, equivalent to 0.005 mg.; or if desired, color standards may be prepared from the standard phosphate solutions, but corresponding to these amounts.

Procedure.

(1) Transfer 0.2 cc. of pus or blood plasma to the narrow test-tube already described.   (2) Add 7 per cent trichloroacetic acid to the 2 cc. mark,[2] close with a small rubber stopper, shake, and centrifuge after a few minutes.   (3) Transfer 0.5 cc. of the clear and colorless fluid, equivalent to 0.05 cc. of the original sample, to the glass-stoppered, graduated, diluting tube of the micro colorimeter.   (4) Add 0.4 cc. of the molybdic-sulfuric acid solution and mix by sharply tapping the lower end of the tube.   (5) Add 0.1 cc. of the diluted stannous chloride reagent, close with the glass stopper, and invert the tube at once.

The color is produced immediately, full intensity being reached in 15 seconds under optimum conditions.   After 1 minute, compare with the permanent color standards or with freshly prepared standards run through in the same manner simultaneously with the unknown.   The unknown is compared with the proper standards in the micro colorimeter by cautious dilution with small quantities of water with the aid of the special pipette.   Any other colorimeter may be used if the unknown and standards are diluted to a suitable volume.

The method of computation using dark Standard B in the micro colorimeter is simple.   Each division on the scale represents 0.1

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[2] If 0.1 cc. of material has been taken make up to the 1 cc. mark.

mg. of phosphorus in 100 cc. of the original material taken for analysis. For example, if the colors match exactly with the meniscus in the diluting tube at 56, then $56 \div 10$ equals 5.6 mg. of phosphorus in 100 cc. of the material taken.

If the color had been matched with the light standard each division would represent 0.05 mg. per 100 cc. and the reading would be divided by 20, thus $56 \div 20$ is 2.8 mg. When working with material relatively low in phosphorus and protein such as spinal fluid, for example, 0.2, 0.25, or 0.5 cc. is made up to 1 cc. only with the trichloroacetic acid. The reading is now divided by 40, 50, or 100 as the case may be.

F = amount of original sample actually used for colorimetric comparison.
S = amount of P or Ca in standard.
R = reading of unknown on graduated scale.

Then P or Ca per 100 c. $= \dfrac{100}{F} \times R \times \dfrac{1^*}{100} \times S.$

For example, if $F = 0.05$ cc., $R = 56$, and $S = 0.005$ mg. of P or Ca, then in 100 cc. original sample

$$P \text{ or } Ca = \frac{100}{0.05} \times 56 \times \frac{1}{100} \times 0.005 = 5.6 \text{ mg. per 100 cc.}$$

With the use of permanent standards a correction should be made by adding 1 per cent for every degree above 21°C., or subtracting below 21°C.

The smallest amount of phosphorus that can be determined in this manner is 0.25 mg. in 100 cc.

Micro Estimation of Calcium.

Two permanent standards are used: A, equivalent to 0.01 mg. of calcium, and B, equivalent to 0.005 mg. If desired, color standards may be prepared for calcium from the standard phosphate solutions, but corresponding to these amounts. An accurately calibrated 0.2 cc. pipette is used, graduated in hundredths and of a bore sufficiently large for thick pus should such be encountered.

Procedure.

Transfer 0.1 cc. of pus or blood plasma with the above pipette to a small platinum dish, rinse the pipette several times with water,

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* 1 unit of the graduated scale.

and add the rinsings to the dish.   Evaporate to dryness, cautiously burn off all organic matter, add a drop or two of concentrated nitric acid, and again ignite in the usual manner.   Care must be taken of course to prevent spattering during the process, which can be completed in about 10 minutes.   After cooling, dissolve the precipitate with 7 per cent trichloroacetic acid in successive small portions of about 0.2 to 0.3 cc. each.   The dish is rinsed down the sides with each portion which is then transferred quantitatively to the narrow test-tube, with the aid of the special pipette drawn to a fine bore at the lower end.   The total volume should not be much over 1 cc.

The calcium may be precipitated from this solution or from 1 cc. of the deproteinized filtrate as obtained in the micro method for

TABLE I.

Mg. of calcium per 100 cc.

	Ashed.	Trichloro-acetic acid.	Trichloro-acetic + phosphoric acid.
Blood serum...............................	11.5	9.5	
" "	10.0	8.0	
" "	10.0	8.5	8.8
" "	12.5	10.4	11.0
Pus, empyema, chest......................	45.6	32.0	38.0
" " " 8 mo. later..........	14.6	13.8	14.2
" osteomyelitis..........................	19.8	17.4	18.9
" "	13.5	12.2	13.1

phosphate determination.   In the latter case the results are somewhat lower, but are generally within 10 per cent of those obtained by the ashing method.   When 0.5 cc. of syrupy phosphoric acid is added to the liter of 7 per cent trichloroacetic acid, the result is better.   This is shown in Table I.

In either case proceed as follows: (1) Add 0.2 cc. of the alkaline sodium phosphate mixture and set aside for 1 hour.   Centrifuge 3 minutes at high speed and discard the supernatant fluid, catching the last drop on a piece of blotting paper.   Allow to drain 1 to 2 minutes and wipe the rim of the tube.   Wash twice with 1 cc. portions of the faintly alkalinized alcohol, rinsing the sides of the tube free from phosphates.   Drain and wipe the rim of the tube after each centrifuging as before.   When no colored indicator is used in

the reagents it is difficult to see the slight precipitate, but this need not cause concern. (2) Add 0.4 cc. of the molybdic-sulfuric acid reagent and dissolve the precipitate, then add 0.5 cc. of distilled water. If desired, 0.9 cc. of a diluted reagent may be used in the proportion of 40 cc. of the molybdic-sulfuric acid to 50 cc. of distilled water. (3) Add 0.1 cc. of the diluted stannous chloride reagent, close the tube with a rubber stopper, and invert at once. After 1 minute, transfer the solution with the aid of the special pipette to the gradu-

TABLE II.

Mg. of calcium per 100 cc.

	Kramer and Tisdall.	Van Slyke and Sendroy.	Micro-colori-metric.
Solution of CaCl₂, containing 10 mg. Ca per 100 cc..................................	10.2		10.0
	9.9		10.2
Solution of CaCl₂, containing 15.1 mg. Ca per 100 cc...............................	15.2	15.0	15.2
	14.8	15.1	15.0
Blood serum (ashed)...........................	11.4	11.5	11.5
" "		9.6	9.5
" "	10.4	10.5	10.5
" " + 10 mg. as CaCl₂ per 100 cc....	20.2		20.4
" "	12.0		12.0
" "		9.0	9.2
" " tetany...........................		5.0	5.1
" " "		6.7	6.8
" "	11.5	11.0	11.3
Pus, empyema, chest..........................	6.4		6.3
" " "	6.0		7.0
" "	45.2		45.6
Spinal fluid, infection.......................	6.0		6.5

ated diluting tube of the colorimeter, and compare with the standards.

The colors are matched in the same manner as described for phosphorus. The computation is exactly similar, except that the final result must be multiplied by 2 if only 0.05 cc. of material has been ashed.

As shown in Table II the method is accurate and yields results comparable with those of the Kramer and Tisdall (11) and the Van Slyke and Sendroy (12) methods.

SUMMARY.

A colorimetric method has been described for the estimation of phosphorus and calcium, based on the selective reduction of phosphomolybdic acid by stannous chloride when definite concentrations of reagents are maintained.

Quantitative micro methods have been described for the estimation of phosphates and calcium in 0.1 to 0.2 cc. of material. As little as 0.05 cc. of pus may be used if only this amount is available.

Newly designed glassware allowing greater accuracy has been added to the micro colorimeter (13). A stannous chloride reagent is described which is more stable than others heretofore used.

Amounts of phosphorus and calcium can be estimated ranging from 0.25 mg. per 100 cc. to 36 mg. and upwards, each individual test representing about 0.00125 to 0.009 mg.

The advantages are greater rapidity in development of color, and a better blue and more intense coloration. The method is more sensitive and permits the detection and determination of a smaller amount of phosphorus and the use of less material than other methods heretofore published.

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THE AVAILABILITY OF DISULFIDE ACIDS AS SUPPLEMENTING AGENTS IN DIETS DEFICIENT IN CYSTINE.*

By BEULAH D. WESTERMAN† with WILLIAM C. ROSE.

(From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.)

In a former communication from this laboratory (Cox and Rose, 1926, *b*) it was shown that synthetic *dl*-β-4-imidazole lactic acid is capable of serving in place of histidine in the diet. The addition of the imidazole derivative to a ration adequate in every respect except as regards its histidine content leads to an immediate resumption of growth at a rate only slightly less rapid than that induced by an equivalent quantity of the amino acid. Inasmuch as this was the first successful attempt, by means of growth experiments, to replace an indispensable amino acid by a non-amino compound, similar investigations with other essential dietary components appeared to be especially interesting and important. As stated elsewhere (Cox and Rose, 1926, *a*), such studies offer a valuable means of discovering what types of chemical changes, particularly of the synthetic sort, may be accomplished by the living organism.

In the present paper we are presenting the results of growth studies in which young animals received diets deficient in cystine, but supplemented with sulfur compounds of more or less close similarity to the amino acid in chemical structure. Cystine is unique among the amino acids in that it is the only well recognized

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* A portion of this communication was presented in abstract before the American Society of Biological Chemists at Rochester, N. Y., April, 1927. See Westerman, B. D., and Rose, W. C., *J. Biol. Chem.*, 1927, lxxiv, p. lxvii.

† The experimental data in this paper are taken from a thesis submitted by Beulah D. Westerman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Physiological Chemistry in the Graduate School of the University of Illinois.

sulfur-containing component of the protein molecule.   Because of this fact it occurred to us that the animal organism might be less exacting in respect to it than in the case of histidine, and be capable of utilizing for growth a variety of derivatives provided they carry the disulfide linkage.   Investigations in several laboratories (Beard, 1925–26; Lewis and Lewis, 1926; Rose and Huddlestun, 1926) have demonstrated conclusively that taurine, which is generally believed to have its origin in cystine, is quite incapable of serving in place of the latter for purposes of maintenance or growth.   Obviously, taurine does not contain the disulfide linkage. Therefore, it does not follow necessarily that negative findings would be obtained in experiments involving the use of compounds more nearly related to the amino acid in structural configuration. Recently, Lewis and Lewis (1927) have shown that the peptides, diglycyl-cystine and dialanyl-cystine, are available for the uses of the organism, but that the diketopiperazine, dialanyl-cystine dianhydride, is not.

<div align="center">EXPERIMENTAL.</div>

The compounds employed in our experiments were dithio-diglycollic acid (I) and β-dithiodipropionic acid (II), both of

$$
\begin{array}{ll}
\text{CH}_2\text{S}\text{—}\text{SCH}_2 & \qquad \text{CH}_2\text{S}\text{—}\text{SCH}_2 \\
\ \ |\qquad\quad | & \qquad\ \ \ |\qquad\quad | \\
\text{COOH}\quad\text{COOH} & \qquad \text{CH}_2\qquad\text{CH}_2 \\
\qquad\ \text{I.} & \qquad\ \ \ |\qquad\quad | \\
 & \qquad\text{COOH}\quad\text{COOH} \\
 & \qquad\qquad\text{II.}
\end{array}
$$
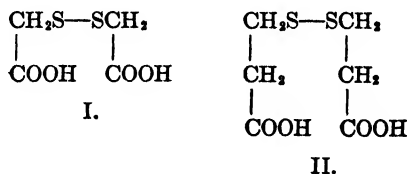
which were synthesized in this laboratory.   The dithiodiglycollic acid was prepared by a modification of the procedure of Claësson (1881).   For this purpose, 20 gm. of commercial thioglycollic acid were dissolved in water, the solution rendered strongly acid with hydrochloric acid, and treated with a little more than the theoretical amount of finely pulverized iodine.   The whole was then heated on the water bath for 10 minutes to accelerate the oxidation, and allowed to stand at room temperature for 3 or 4 hours.   The resulting dithiodiglycollic acid was removed by extracting the mixture several times with alcohol-free ether.   The ether extracts were combined, freed from excess iodine by shaking

with a solution of sodium thiosulfate, and evaporated to crystallization. The acid was purified by dissolving in alcohol-free ether, and precipitating with 2 volumes of petroleum ether. The product separated as a white solid which, after drying in the air, was found to melt at the theoretical point of 100°C. Analyses showed the presence of 35.22 per cent of sulfur as compared with the theoretical of 35.16 per cent. The yield of the pure material amounted to 9.0 gm.

The β-dithiodipropionic acid was prepared essentially according to the directions of Biilmann (1905), by treating β-chloropropionic acid with potassium xanthate, decomposing the condensation product with ammonia, and oxidizing the resulting β-mercaptopropionic acid with iodine. A yield of approximately 18 per cent calculated on the basis of the original β-chloropropionic acid was secured. After recrystallizing the acid from hot water, the pure white material melted at the theoretical point of 155°C., and had a sulfur content of 30.60 per cent as compared with the calculated value of 30.48 per cent.

Four litters of rats of our own rapidly growing stock were employed in the investigation. The basal diet was a slight modification of one used by Sherman and Merrill (1925), and had the following composition:

	per cent
Whole milk powder	16.66
Corn-starch	81.67
Sodium chloride	1.67

These authors have shown that such a ration, when supplemented with yeast as a source of vitamin B, and 0.20 per cent cystine, supports long continued, steady growth in rats at a rate about two-thirds the normal maximum. In the absence of added cystine, the increase in body weight is much less rapid. In all of our experiments, except those upon Litter D, we have fed separately 50 mg. of commercial yeast extract and 4 drops of cod liver oil to each rat daily in order to insure the presence of adequate quantities of the vitamins. Each member of Litter D received 75 mg. of Vegex daily as a source of vitamin B. For the animals which received cystine or one of the disulfide acids, the substance in question replaced an equivalent amount of starch. The

quantities of the supplementary sulfur compounds employed
were:

<div align="right">per cent</div>

Cystine... 0.20
Dithiodiglycollic acid................................. 0.16
β-Dithiodipropionic acid.............................. 0.18

The results of the experiments are presented graphically in
Charts I to IV.   The curves show conclusively that neither of
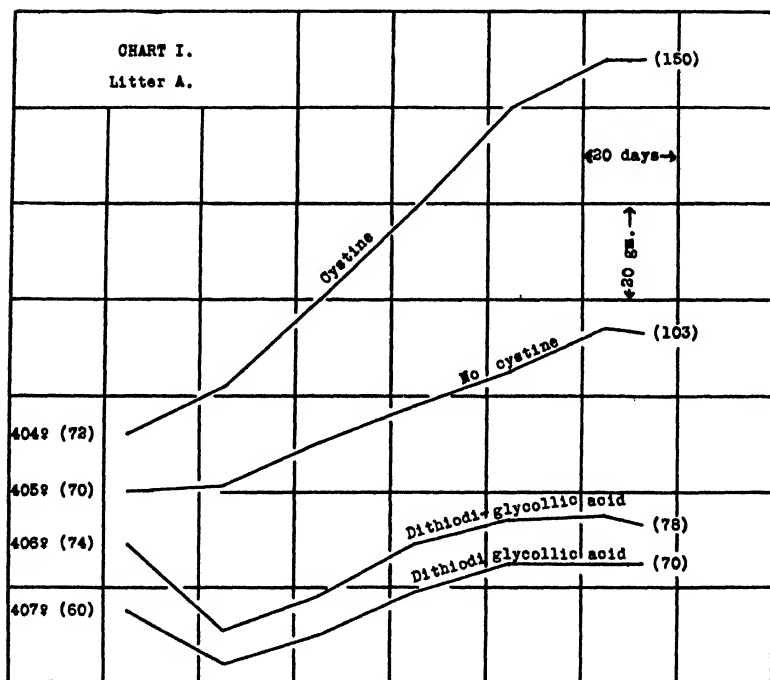
CHART I.   In all of the charts the numbers in parentheses signify the
initial and final weights of the rats.

the synthetic products is capable of serving to the slightest extent
in place of cystine.   Even β-dithiodipropionic acid, which is very
closely related structurally to cystine, fails entirely to improve the
nutritive condition of animals suffering from cystine deficiency.
Evidently, the presence of the disulfide linkage is not the only
structural prerequisite necessary to render a compound capable of
replacing cystine.

CHART II.

Litter B.

Cystine

Cystine

←30 days→

20 gm.↑↓

414♂ (80)

415♂ (94)

416♂ (94)

417♂ (96)

418♀ (88)

419♂ (86)

421♂ (96)

420♂ (96)

No cystine

(112)

Dithiodi-glycollic acid (96)

Dithiodi-glycollic acid (72)

Dithiodi-glycollic acid

(70)

Dithio- diprop. acid (86)

Dithio- diprop. acid (92)

(150)

(150)

CHART II.

CHART III.

Litter C.

Cystine (116)

Cystine (100)

←30 days→

20 gm↑↓

436♂ (75)

437♀ (62)

438♀ (65)

439♂ (62)

430♂ (70)

431♂ (74)

432♀ (66)

No cystine (72)

No cystine (72)

Dithiodi- prop. acid (88)

Dithio- diprop. acid (78)

Dithio- diprop. acid (78)
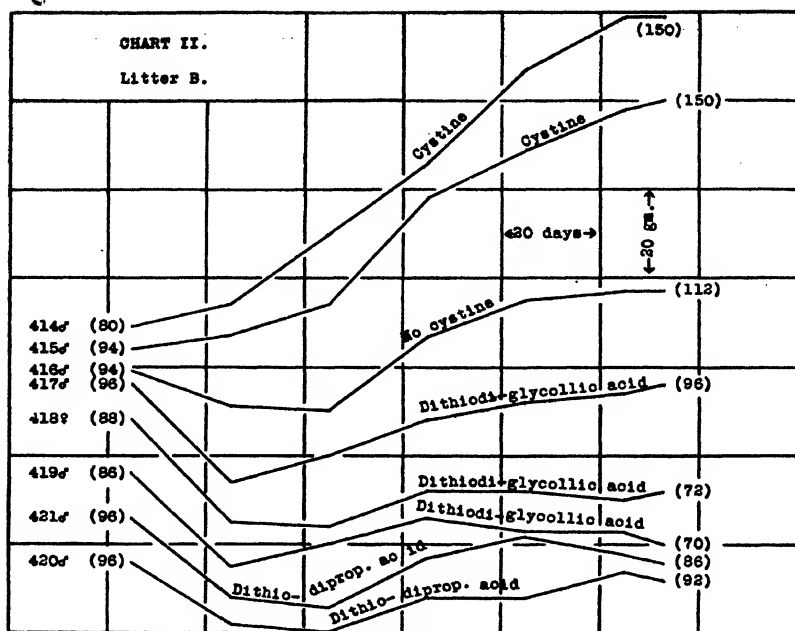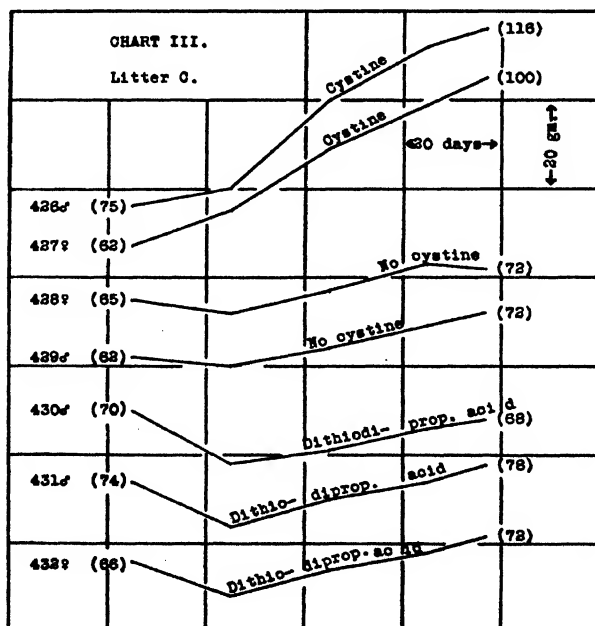
CHART III.

An examination of Charts I to III reveals the fact that all of the animals which received the synthetic compounds lost weight quite rapidly during the first 20 days of the experiments; more rapidly, in fact, than did their litter mates upon the basal diet
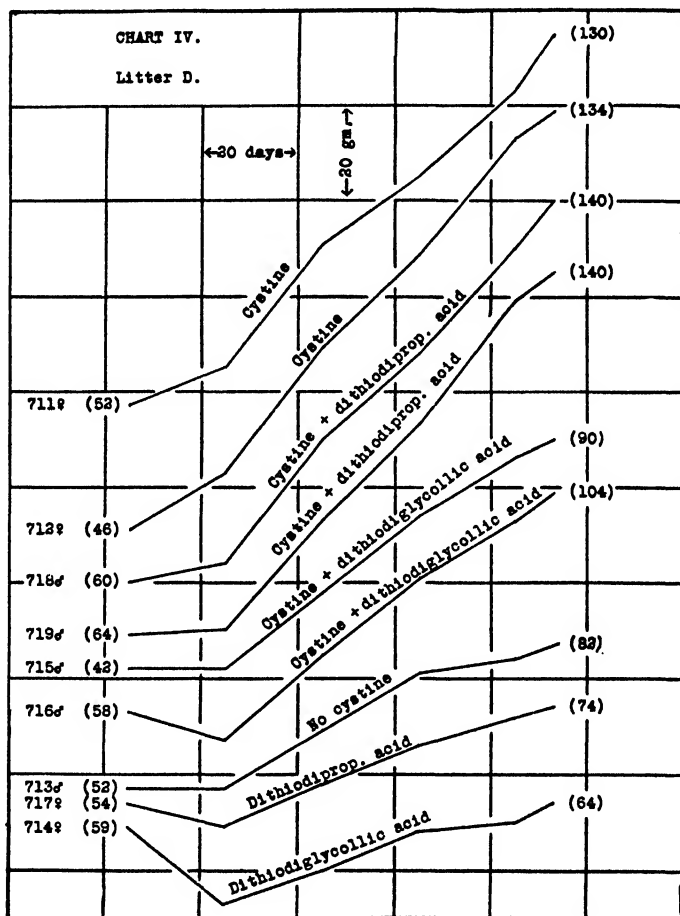
CHART IV.

without added cystine. In order to determine whether the synthetic products exert a toxic influence which interferes with growth the experiments outlined in Chart IV were carried out. Two of the rats of this litter (Litter D) received both cystine and dithio-

diglycollic acid, and two received cystine and β-dithiodipropionic acid. The graphs show that the presence in the ration of β-dithio-

TABLE I.

Food Consumption of Experimental Animals.

Litter.	Rat No.	Diet.	Average daily food consumption.
			gm.
A	404	Basal + cystine.	8.1
	405	"	6.5
	406	" + dithiodiglycollic acid.	5.5
	407	" + " "	4.9
B	414	Basal + cystine.	7.8
	415	" + "	7.2
	416	"	6.5
	417	" + dithiodiglycollic acid.	5.4
	418	" + " "	3.9
	419	" + " "	3.7
	420	" + dithiodipropionic "	5.2
	421	" + " "	5.2
C	426	Basal + cystine.	6.9
	427	" + "	6.0
	428	"	5.1
	429	"	4.8
	430	" + dithiodipropionic acid.	4.4
	431	" + " "	5.0
	432	" + " "	4.8
D	711	Basal + cystine.	7.5
	712	" + "	7.5
	713	"	6.0
	714	" + dithiodiglycollic acid.	4.2
	715	" + cystine + dithiodiglycollic acid.	4.6
	716	" + " + " "	5.1
	717	" + dithiodipropionic acid.	5.1
	718	" + cystine + dithiodipropionic acid.	6.9
	719	" + " + " "	7.0

dipropionic acid does not prevent relatively rapid growth provided cystine also is supplied. Rats 718 and 719, which received both the amino acid and the synthetic derivative, made practically

as good growth as did Rats 711 and 712 which received cystine alone. On the contrary, Rats 715 and 716 which received dithiodiglycollic acid and cystine gained little more than half as rapidly as did the controls, Rats 711 and 712. Evidently the presence of dithiodiglycollic acid in the food exerts an inhibitory influence upon growth. Whether this is to be attributed to a direct toxicity of the compound, or to an indirect alteration in food consumption, is uncertain. A comparison of the data summarized in Table I evinces the fact that most of the animals which received the synthetic compounds ingested less food than did their litter mates.

We are now synthesizing other derivatives more closely related to the amino acid in chemical structure with the expectation of determining whether they are more satisfactory supplementing agents for cystine-deficient rations.

SUMMARY.

Synthetic dithiodiglycollic acid and β-dithiodipropionic acid have been fed to rats upon diets deficient in cystine in order to determine whether the substances in question can replace the amino acid for purposes of growth. The results show conclusively that neither is capable of serving in place of cystine. Despite the close similarity in chemical structure of cystine and β-dithiodipropionic acid the two behave entirely differently in the animal organism.

Evidence is presented indicating that the incorporation of dithiodiglycollic acid in the ration leads to a subnormal growth even when cystine is also furnished. Apparently the synthetic acid is either toxic or in some other fashion exerts an inhibitory influence upon the growth processes. The food consumption of rats receiving dithiodiglycollic acid is considerably less than that of other animals upon similar diets without the synthetic product. On the other hand, the presence of β-dithiodipropionic acid in the food produces little if any interference with growth provided adequate amounts of cystine are supplied. Rats receiving both cystine and β-dithiodipropionic acid make practically as satisfactory gains as their litter mates upon diets containing cystine alone.

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A MODIFIED METHOD FOR THE ESTIMATION OF TOTAL CREATININE IN SMALL AMOUNTS OF TISSUES.

By WILLIAM C. ROSE, OSCAR M. HELMER, AND ALFRED CHANUTIN.

(*From the Laboratory of Physiological Chemistry, University of Illinois, Urbana.*)

In a study of the distribution of total creatinine (creatine plus creatinine) in rats it was necessary to employ a method for the estimation of this substance which would be applicable to 1 gm. quantities of the tissues. Most of the available methods for tissue creatine require considerably more material than can be obtained readily from small animals.

The two general procedures employed for the removal of creatine from tissues are (*a*) extraction, and (*b*) disintegration by means of acid. Perhaps both procedures are open to criticism on theoretical grounds. In the case of the first, the difficulty of knowing when the extractions are complete, the large volumes of fluid which inevitably result, and the time-consuming nature of such determinations are very undesirable qualities of a method to be applied in a large number of experiments. Furthermore, as pointed out by Folin and Buckman (1914), extraction methods are apt to give minimum values. On the other hand, the acid disintegration principle has been criticized (Janney and Blatherwick, 1915) on the ground that certain tissues, particularly those of dogs, give too high values following treatment with heat and acid. This statement has been questioned by Baumann and Hines (1916) who obtained comparable results by the two procedures when applied to dog tissues. On the whole, acid disintegration appears to us to be less objectionable than extraction, and for this reason is employed in the following method.

The principle of the method which was finally adopted is very

similar to that suggested independently by Baumann (1914) and Folin (1914), but differs in that we employ tungstic acid as a clarifying agent following the disintegration of the tissues with sulfuric acid. Numerous experiments were made in order to discover the optimum conditions as regards strength of acid, time of heating, and reaction for the tungstic acid treatment. It seems unnecessary to describe in full these preliminary tests. The method in detail is as follows:

The fresh tissues or organs are removed as promptly as possible after the death of the animal. If the material is abundant, as in the case of the muscle, it should be run through a meat chopper before sampling. With small organs cutting with scissors is sufficient. In any event approximately 1 gm. portions (smaller quantities may be used if necessary) are dropped into previously weighed 50 cc. glass-stoppered Erlenmeyer flasks. The latter are kept tightly closed to prevent evaporation of moisture until they with their contents have been weighed again. Having thus determined the weights of the samples, each is treated with 20 cc. of 2 N sulfuric acid. The flasks are covered with tin-foil, and heated for 45 minutes in an autoclave at 15 pounds pressure. After cooling, each solution is transferred to a 100 cc. volumetric flask with the aid of 40 to 50 cc. of water, treated with 18 cc. of 2 N sodium hydroxide, and 5 cc. of 10 per cent sodium tungstate $(Na_2WO_4 \cdot 2H_2O)$. The mixture should now be faintly acid to Congo red. After making up to volume with water and thoroughly shaking, the flask is allowed to stand for 5 minutes and the contents filtered.

The remainder of the method is carried out just as in the Folin-Wu (1919) procedure for the determination of creatinine in blood. 10 cc. of the clear filtrate are treated with 5 cc. of alkaline picrate prepared by mixing 25 cc. of a saturated solution of purified picric acid and 5 cc. of 10 per cent sodium hydroxide. At the same time 20 cc. of a standard creatinine solution are treated with 10 cc. of the alkaline picrate. After standing for 8 minutes the color comparison is made as usual with the standard at 20 mm. In the analysis of voluntary muscle a convenient standard is one containing 0.8 or 1.0 mg. of creatinine per 20 cc. For other tissues more dilute standards must be employed.

The colorimetric comparisons should be completed, especially

in the analysis of liver which may be rich in glycogen, within 12 or 13 minutes from the time the color is developed. On longer standing too high values may result, probably because of the production of chromogenic substances by the action of the acid on glucose. If the analysis is completed within the time specified no detectable error occurs even though the tissue is taken from an animal previously fed a high carbohydrate diet to increase the store of glycogen. We do not recommend the method for blood inasmuch as the various procedures described in the literature involving the precipitation of the proteins preliminary to the acid treatment are simpler and perhaps more reliable. On the other hand, probably no available method gives entirely correct values for total creatinine in blood (cf. Hunter and Campbell, 1917; Behre and Benedict, 1922). It follows, therefore, that determinations of creatine in tissues may be rendered slightly inaccurate by the blood present in the material analyzed. We are convinced that the method outlined above is at least as trustworthy as any hitherto recommended, and possesses the additional advantages of convenience and applicability to small amounts of tissues.

The tungstic acid employed in our method serves primarily as a clarifying and decolorizing agent rather than as a precipitant. The amount of precipitable material is small after autoclave hydrolysis. Indeed, in muscle analyses the tungstate treatment probably could be omitted altogether without materially altering the creatinine results. But in the analyses of the more vascular organs, such as the liver and kidneys, considerable pigmentation occurs. When the samples are removed from the autoclave they have a reddish brown color due to the disintegration of hemoglobin. It is essential that this color be removed, and the tungstic acid is employed chiefly for this purpose. Incidentally, some nitrogenous material other than creatinine is precipitated also, as is shown by determinations of non-protein nitrogen in the filtrates before and after the tungstate treatment.

For successful decolorization it is important that the reaction of the solution be carefully controlled, inasmuch as traces of excess alkali and larger quantities of excess acid dissolve some of the pigment. With 1 gm. quantities of the tissues 1.8 cc. of 2 N sodium hydroxide usually will reduce the acidity to the optimum concentration for decolorization. It must be borne in mind, how-

ever, that the volume of alkali required will depend somewhat upon the amount of tissue employed for the individual determination, and upon the quantity of carbonate present in the sodium tungstate. If the filtrate contains more than a trace of coloring matter the reaction for the tungstate treatment is incorrect, and more or less alkali, as may be necessary, must be used. The less vascular tissues and organs do not require such careful control of

TABLE I.

Comparative Data Secured with Accepted Methods and with the Proposed Procedure.

Expressed in mg. of creatine per 100 gm. of tissue.

Tissue.	Baumann and Hines.	Myers and Fine.	Proposed procedure.
Beef muscle.....................	432		420
" "		458	452
Dog "		380	392
" "		351	342
Rabbit muscle*..................	612	616	615
" "		580	577
" "		574	573
" "		572	565
" "		550	560
" "		510	503
" "		493	483
Rat muscle......................		467	471
" "		472	468
" "		467	463
" carcass†		348	341

* This animal had been used in an experiment, which probably accounts for the high values.

† All of the tissues with exception of the skin and viscera.

the reaction, and under the conditions outlined above never yield colored filtrates. Moreover, with all tissues the final results are the same whether the tungstate is added to a slightly acid solution, or to a neutral solution which is subsequently treated with an appropriate quantity of acid. In view of this fact we have adopted the former procedure as being the simpler for the purpose in question.

One other detail of the method requires explanation. The

reaction recommended for decolorization is one which is distinctly but not strongly acid to Congo red. This is more acidic than the reaction employed by Folin and Wu in precipitating proteins from blood. Probably as a result of the higher acidity in our method the resulting solutions tend to foam somewhat when shaken before filtration. This need give no concern whatever. As stated above we are using the tungstic acid primarily as a

TABLE II.

Recovery of Creatine Added to Muscle and Liver.

Expressed in mg. of creatine per 100 gm. of tissue.

Tissue.	Creatine content.	Creatine added.	Creatine found.	Added creatine recovered.
				per cent
Beef muscle.........	327	86	417	104.7
" "	316	129	444	99.2
Rabbit muscle.......	506	300	805	99.7
" "	483	500	989	101.2
" "	517	500	1035	103.6
" "	506	700	1220	102.0
" liver..........	46.5	168	209	96.7
" "	46.5	422	474	101.3

TABLE III.

Total Creatinine (Creatine plus Creatinine) Content of Tissues of Rabbits.

Expressed in mg. of creatine per 100 gm. of tissue.

Rabbit No.	Muscles of leg.	Heart.	Brain.	Liver.	Spleen.	Kidneys.	Testes.
1	515	223	121	27	27	19	188
2	517	217	106	24		20	194
3	492	193	114	24	27	19	182

clarifying and decolorizing agent and only secondarily as a precipitant. Our first consideration, therefore, has been to adopt the most favorable conditions for the purpose in question.

We have used the above method in several hundred determinations during the past 2 years, and have found it convenient and accurate. Determinations upon the muscles of dogs, rabbits, and rats have given comparable results with our method and the methods of Folin (1914), Myers and Fine (1915), and Bau-

mann and Hines (1916). Data of this sort are summarized in Table I. Analyses of tissues with and without the addition of known amounts of creatine have yielded excellent recoveries, as shown in Table II. In Table III are presented the results of distributional analyses upon three rabbits. The uniformity of the figures is striking, and the values are quite close to those given in the literature for this species.

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A STUDY OF THE EFFECT OF CREATINE ON GROWTH AND ITS DISTRIBUTION IN THE TISSUES OF NORMAL RATS.

BY ALFRED CHANUTIN.

(*From the Laboratories of Physiological Chemistry, University of Illinois, Urbana, and University of Virginia, University.*)

The idea that creatine might serve as a food was first advanced by Folin (1). The evidence for this suggestion was based on the failure to recover or account for small amounts of ingested creatine. Benedict and Osterberg (2), in a series of experiments with dogs, demonstrated conclusively that ingested creatine is partially retained, and apparently utilized by the organism, and that dogs fed creatine showed an increase in weight and a positive nitrogen balance. It was shown by Chanutin(3) that a positive nitrogen balance follows creatine feeding in man. It has been suggested (2, 3) that creatine may be responsible for the gains in weight noted in the experimental subjects.

Since creatine occurs in practically all the tissues of the body, it may be assumed that it plays a rôle in normal metabolism. Despite the general distribution of this substance, there is evidence that the muscles and liver are chiefly concerned in its metabolism. These tissues have been investigated principally in relation to the storage of creatine. In many of the experiments reported in the literature, data were obtained from subjects suffering with obscure pathological conditions such as muscular dystrophy or hepatic diseases. In some cases these abnormal conditions have rendered interpretation difficult or impossible.

Mellanby (4) has presented evidence which indicates that the liver is intimately associated with creatine metabolism. This investigator found that the muscle creatine of the chick embryo appears and increases synchronously with the development and growth of the liver. Furthermore, he states that although the cross-striated muscles of the invertebrates are histologically identical with those of the vertebrates, creatine is absent from the tissues of the lower forms because there is no organ present which corresponds to the liver of the vertebrates. Mellanby has added more data to support his contention by studying the creatine and creatinine output in people suffering from liver affections. He found that creatinine excretion is diminished in certain types of hepatic disease, and has suggested that the liver is responsible for the formation of creatinine. Chisholm (5), after

examining the creatine content of livers obtained at autopsy, reported a reduction in creatine in cases of malignant disease.

Evidence contradictory to Mellanby's hypothesis has been submitted by several workers. Towles and Voegtlin (6) observed no difference in the metabolism of creatine in Eck fistula and normal dogs. These authors concluded that the liver performs no important rôle in creatine metabolism. Folin and Denis (7) have stated that: "The liver has no special function to perform in connection with the creatinine formation." No direct evidence supporting this conclusion was given. After perfusing the liver of the dog with Ringer's solution containing creatine, Scaffidi (8) observed no formation of creatinine. According to Paton and Mackie (9), exclusion of the liver in the goose has no effect on creatine metabolism.

Since voluntary muscle is rich in creatine, this tissue must be considered of fundamental importance in any discussion of creatine metabolism. Attempts have been made to study the fate of ingested and injected creatine by analyzing muscle tissue. Myers and Fine (10) were able to find an appreciable increase in the creatine content of rabbit muscle after subcutaneous injections of creatine. Folin and Denis (7, 11) found an increase in muscle creatine in cats after intestinal and intravenous injections of creatine.

In an effort to learn more about the capacity of the tissues for creatine storage, and the possible relationship of this substance to increase in body weight, the following experiments were undertaken.

EXPERIMENTAL.

The white rat served as the experimental subject. The composition of the diets used in the experiments is shown in Table I. In addition to the experimental ration each rat received daily 50 mg. of Yeast Vitamine (Harris) as a source of vitamin B, and 6 drops of cod liver oil to supply the fat-soluble vitamins. Diet 31, when supplemented with vitamins, is adequate in every respect. Diets 32 and 33 are similar in composition with the exception that they contain 0.67 and 2.67 per cent of creatine, respectively. The creatine used in the experiments was prepared by double recrystallization of a crude commercial product. Nitrogen determination upon this product gave results closely approximating the theoretical values.

At definite intervals the rats were killed and the tissues were analyzed for creatine in order to determine the extent of creatine storage. Any significant increase in the creatine content of the tissues of creatine-fed animals would indicate either a creatine

reservoir or would reveal a site important in the metabolism of this substance. To obtain the tissues for analysis, the rats were anesthetized with chloroform. While the heart was still beating the jugular vein and carotid artery were severed and the animal bled to death. The muscles of the hind legs were dissected free and immediately ground in a small meat chopper. The remaining organs were removed at once and cut into small pieces with scissors. These tissues were immediately placed in stoppered containers to avoid evaporation. Within an hour after a rat was killed the tissues were placed in sulfuric acid. After weighing the tissues, the analysis for creatine (total creatinine) was made by the method of

TABLE I.

Composition of Diets.

	Diet 31.	Diet 32.	Diet 33.
	gm.	*gm.*	*gm.*
Casein...........................	18.0	18.00	18.00
Dextrin..........................	36.0	35.33	33.33
Sucrose..........................	15.0	15.00	15.00
Lard.............................	25.0	25.00	25.00
Salt mixture*....................	4.0	4.00	4.00
Agar.............................	2.0	2.00	2.00
Creatine.........................		0.67	2.67
Total.........................	100.0	100.00	100.00

* Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

Rose, Helmer, and Chanutin (12). It is assumed that only a very small amount of creatinine is present in the tissues studied.

Effect of Creatine on Growth of Young Rats.—The average daily food and creatine consumption and the average body weight changes of the rats are summarized in Table II. It will be noted in Table II that half of the animals in each group were sacrificed at the end of a month in order to determine the creatine distribution in the tissues. The data obtained in the remaining animals are sufficient to demonstrate that the addition of creatine to an adequate ration does not affect the growth of rats. The food consumption data show that the daily calorific intake was fairly constant for the three diets studied. Furthermore, within each

TABLE II.

Food and Creatine Consumption and Body Weight Changes.

Rat No.	Average daily food consumption.	Average daily creatine consumption.	Initial.		Final.		Litter.
			Age.	Body weight.	Age.	Body weight.	
Normal diet.							
	gm.	gm.	days	gm.	days	gm.	
457	6.8	0	36	76	76	119	58
459	6.8	0	35	94	77	147	61
465	6.8	0	35	92	81	154	64
473	5.5	0	31	55	79	110	57
451	6.8	0	36	80	72	122	58
463	7.5	0	35	79	102	158	61
468	6.7	0	35	80	103	153	64
472	6.4	0	34	78	105	140	51
453	6.7	0	36	77	106	154	58
476	6.6	0	31	63	103	157	57
Low creatine diet.							
450	5.9	0.040	36	71	75	100	58
458	7.2	0.049	35	90	77	150	61
467	7.3	0.049	35	94	81	167	64
475	5.6	0.038	31	64	79	112	57
452	6.6	0.045	35	82	73	121	58
462	7.6	0.051	35	81	102	166	61
469	6.7	0.045	34	77	103	145	51
456	6.1	0.040	36	77	106	131	58
470	6.7	0.045	34	82	105	159	51
474	6.0	0.040	31	57	104	128	57
High creatine diet.							
455	6.3	0.168	36	70	76	117	58
460	7.1	0.190	35	97	77	151	61
464	6.2	0.168	35	92	81	151	64
477	5.8	0.156	31	58	79	120	57
449	6.5	0.173	36	83	72	123	58
461	7.4	0.198	35	79	102	157	61
466	6.3	0.170	35	80	103	148	64
454	6.7	0.181	36	76	106	152	58
471	6.8	0.181	34	78	106	168	51
478	6.2	0.172	31	62	105	156	57

group of animals the daily creatine intake varied but little. It must be stated, however, that no definite conclusion can be drawn concerning the function of creatine as an anabolite, since the period of experimentation is comparatively short.

Effect of Creatine Feeding upon Creatine Content of the Tissues of Rats.—Tables III and IV contain data upon the creatine concentration in the tissues and organs of normal rats and of creatine-fed rats. The experimental data are given in detail since there are no comparable figures in the literature dealing with the creatine concentration in the tissues of rats. The rats were divided into two groups; *i. e.*, the first four rats in each series were killed about a month after the experiments had begun; the remaining animals were allowed to live a month longer. With the exception of the creatine content of the liver, the data obtained with these two groups varied very little.

The figures in Tables III and IV show that the ingestion of relatively large amounts of creatine over a long period of time does not affect creatine concentration in the heart, brain, and testes. The concentration of creatine in these organs is strikingly constant in individual rats fed on the normal and on creatine-containing diets.

The creatine concentration of the kidneys, involved in the excretion of this substance, is increased slightly in the animals fed on a high creatine diet. This result is not surprising since it is known that a very large proportion of creatine is excreted by the kidney when the experimental subject is on a high creatine diet. There is practically no difference in the creatine content of the kidneys of rats fed on normal and of those fed on low creatine diets.

Although there is a slight increase in the average creatine concentration in the muscles of the animals fed on a high creatine diet, the difference noted can be considered only as suggestive. It is certain that no assumption can be made respecting the muscles as a creatine reservoir under the conditions of these experiments.

The liver seems to be the only organ of animals fed on a high creatine diet in which there is a significant increase in creatine concentration. It will be noted that the livers of the younger rats (Nos. 455, 460, and 464) on the high creatine diet have a comparatively high creatine concentration. No reasons can be postulated at present for the unusual values noted in these animals.

TABLE III.

Creatine Content of Rat Tissues as Influenced by Feeding of Creatine.

Rat No.	Duration of experiment.	Creatine content of:					
		Heart.		Brain.		Testes.	
		Total.	Per gm.	Total.	Per gm.	Total.	Per gm.
	days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
				Normal diet.			
457	40	0.75	1.67	1.95	1.37		
459	42	0.79	1.62			4.11	2.83
465	46	0.92	1.95	1.92	1.28	5.17	2.83
473	48	0.58	1.64	1.70	1.20	3.25	2.67
463	67	0.96	1.81	1.90	1.29		
468	68	0.89	1.73	1.91	1.23		
472	71	0.94	1.75	1.94	1.28	5.17	2.92
453	70	0.88	1.80	2.11	1.42		
476	72			1.96	1.30	5.00	2.81
Average........		0.84	1.74	1.92	1.29	4.54	2.81
				Low creatine diet.			
450	39	0.61	1.53	1.68	1.31		
458	42	0.81	1.63	1.96	1.31	3.84	2.96
467	46	1.00	1.88	2.04	1.29	5.16	2.66
475	48	0.69	1.88	1.88	1.27	3.20	2.72
462	67	1.05	1.81	1.82	1.22		
469	69	0.88	1.94	1.96	1.27		
456	70	0.87	2.02	1.93	1.29		
470	71	0.96	1.94			5.16	2.88
474	73	0.85	1.85	2.09	1.32		
Average........		0.86	1.83	1.92	1.28	4.34	2.78
				High creatine diet.			
455	40	0.73	1.76	2.05	1.50		
460	42	0.86	1.69	2.18	1.38	4.35	2.55
464	46	0.94	2.05	1.94	1.31	5.27	2.72
477	48	0.73	1.67	1.83	1.29	2.69	2.91
461	67	0.95	1.79	1.91	1.23		
466	68	0.88	1.76	1.97	1.31		
454	70	0.90	1.77	1.90	1.30		
471	72	1.05	1.98	1.98	1.30	5.65	2.93
478	74	0.98	1.75			5.05	2.92
Average........		0.89	1.80	1.97	1.33	4.68	2.81

TABLE IV.

Creatine Content of Rat Tissues as Influenced by Feeding of Creatine.

Rat No.	Duration of experiment.	Creatine content of:				Muscle per gm.
		Liver.		Kidneys.		
		Total.	Per gm.	Total.	Per gm.	
Normal diet.						
	days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
457	40		0.36	0.43	0.42	4.60
459	42	1.66	0.28	0.73	0.52	4.78
465	46	3.69	0.59	0.48	0.34	4.50
473	48	1.22	0.27	0.48	0.49	4.38
463	67	1.79	0.34	0.86	0.68	4.50
468	68	1.75	0.33	0.44	0.37	4.60
472	71	1.73	0.29	0.87	0.64	4.26
453	70	1.42	0.29	0.43	0.39	4.62
476	72	1.39	0.25	0.50	0.32	4.23
Average..........		1.83	0.33	0.58	0.46	4.49
Low creatine diet.						
450	39	0.92	0.33	0.44	0.45	4.54
458	42	2.26	0.37	0.68	0.50	4.61
467	46	4.60	0.68	0.70	0.44	4.68
475	48	1.54	0.38	0.65	0.60	4.62
462	67	1.92	0.33	0.53	0.42	4.50
469	69	1.83	0.37	0.56	0.52	4.41
456	70	1.39	0.30	0.54	0.50	4.22
470	71	1.98	0.35	0.70	0.44	4.71
474	73	1.24	0.28	0.58	0.57	4.62
Average.........		1.96	0.37	0.60	0.49	4.54
High creatine diet.						
455	40	10.05	2.14	1.30	1.24	4.92
460	42	6.92	1.20	1.23	0.97	4.83
464	46	6.90	1.11	0.74	0.56	4.82
477	48	3.44	0.72	0.74	0.61	4.57
461	67	4.18	0.70	0.73	0.52	4.50
466	68	4.25	0.83	0.84	0.74	4.74
454	70	4.13	0.79	0.51	0.43	4.30
471	72	2.41	0.38	0.73	0.50	4.88
478	74	1.89	0.34	1.36	0.92	4.62
Average..........		4.91	0.91	0.91	0.72	4.66

DISCUSSION.

There are numerous data on record to indicate that ingested creatine is retained by the body. Regardless of the amount of creatine ingested by the organism, it appears that there is always a deficit in the creatine balance which has not thus far been satisfactorily explained. It is generally believed that retained creatine "may be deposited unchanged in the tissues, especially in the muscles, or it may be utilized in the synthesis of other substances. There is no doubt at all that the first of these alternatives can be realized" (13). The results obtained in these experiments would certainly lead one to the conclusion that this substance, if retained in the muscles, is stored as such in only slight amounts. Furthermore, if an increase in the creatine concentration of a tissue is to be considered an index of its metabolic activity, it would appear that the rôle of muscles in the conversion of creatine to other substances is rather unimportant.

No definite idea can be obtained from the existing experimental data concerning the relationship of the liver to creatine metabolism. It has been argued (4) that creatinine is continually being formed in the liver, carried to the muscles, and there changed to creatine and stored. This storage is supposed to continue until the muscles are saturated, whereupon any excess creatinine is eliminated. It must be pointed out that the evidence on which this theory is based is not strong. However, the proof that the liver plays no part in creatine metabolism is not particularly convincing. Eck fistula dogs have been used as a type of experimental animal in which it is assumed that the liver could play a minor part in metabolism. It is generally conceded by physiologists that this is not strictly true. Nevertheless, results obtained with these animals have been interpreted as evidence of liver deficiency. Clinical data obtained in hepatic diseases can only be considered as suggestive.

Mendel and Rose (14) advanced the theory of a possible relationship between carbohydrate and creatine metabolism. These authors suggested that any interference with the glycogenic function of the liver, or an inability of the organism to burn sugar properly leads to creatinuria. It is well known that drugs such as phosphorus and hydrazine, which act directly on the liver cells, always cause an appearance of creatine in the urine. These facts

certainly suggest an intimate relationship between the liver and creatine metabolism.

The data obtained in this investigation point conclusively to the liver as the only organ in which there is a definite and marked increase in creatine. There is the possibility that the liver may act as an excretory organ for creatine, but this is very unlikely. From our data it appears logical to assume that the liver plays an important rôle in creatine metabolism. It is further suggested that this relationship may be independent of the carbohydrate metabolism of the organism, since a significant increase in the creatine concentration of the liver is found in normal animals.

SUMMARY.

The feeding of adequate diets containing creatine (0.67 and 2.67 per cent) to young rats for a period of 2 months has no effect on the growth curve.

The average creatine concentrations for the organs of normal albino rats are as follows: muscle 0.449, testes 0.281, heart 0.174, brain 0.129, kidney 0.046, and liver 0.033 per cent.

The liver is the only organ in which a significant increase in creatine concentration was noted in animals fed on creatine-containing diets.

A possible relationship is suggested between the liver and creatine metabolism, which is independent of carbohydrate metabolism.

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ON THE HYDROGEN ION DETERMINATION OF NORMAL SALIVA.

By MARJORIE HENDERSON AND JOHN A. P. MILLET.

(*From the Austen Riggs Foundation, Stockbridge, Massachusetts.*)

For a number of years we have noted in many of our patients a strongly acid reaction of their saliva to litmus paper. In order to determine whether or not this clinical observation has any value as an indicator of emotional imbalance, or temporary emotional stress, it seemed wise first to investigate the reaction of saliva collected from normal individuals under the varying conditions of an average working day. The following study was carried out with this object in view, the subjects being drawn chiefly from the professional and secretarial staff of the Austen Riggs Foundation.

A brief summary of earlier work and the methods used has been compiled by Starr (1922).

We have selected the colorimetric pH method as best suited for our work and later checked this with the electrometric method.

From our work, though necessarily done on a limited number of individuals, the constancy of the results and the sharp relationship of the pH of saliva to the ingestion of food make it seem probable that there is a definite correlation of the pH of saliva with the ingestion of food and the day's work. If breakfast is finished at 10 instead of at 8 o'clock, then the pH of saliva at 11 will correspond with the pH found 1 hour after a meal, and not with the pH obtained at 11 when breakfast is at 8. In our experience also we find that while the pH of saliva varies in different individuals and to a limited degree in the same individual, the diurnal curve tends always to follow the same general outline.

Procedure.

All glassware (such as test-tubes and vials) was kept filled with a solution of brom-thymol blue of pH 6.9. If any alteration of the

solution occurred the article in question was not used.   All pipettes were rinsed in a similar solution just before using.   The thistle tube used for collection of the sample was always rinsed in fresh tap water just before use.

The salivary specimens were collected by means of a thistle tube under 1 cc. of oil in a vial 1 cc. in diameter.   Every determination was made immediately after expectoration, except in the case of the early morning samples.   For a number of days these were also analyzed immediately and then checked an hour later to see whether there was any change in reaction from standing.   1 cc. of saliva was diluted with 9 cc. of distilled water, which had been tested and found to have a pH of 6.9.   (To save time this was usually made up in bulk and kept on hand.)   Clark and Lubs hydrogen-ion standards were used for all determinations.

Since there had been considerable discussion in previous work regarding the possible loss of carbon dioxide and the necessity of collecting saliva under oil, we first investigated this problem and found that a person could readily collect approximately 1 cc. of saliva and in many cases much more before expectorating.   Tests made immediately after the sample was taken, both under oil and without oil, showed no variation in the results, so that we concluded that, if the tests were made immediately after collection of the sample, oil was unnecessary.   As there was always the possibility of being called away, however, we made use of oil generally throughout our work.   Standing for 1 hour did not seem to alter the results colorimetrically, but longer periods of standing resulted in a more acid saliva, though in some cases there was little change to be noted.

It seemed to be the general conclusion that chewing paraffin or pure rubber increased the salivary pH.   We, therefore, avoided this possibility of altering the actual pH of the saliva and collected it normally without any conscious effort or masticating activity. Our routine was as follows: Give first sample on rising in the morning before activity, second sample just before breakfast, then clean teeth (cleaning the teeth always raised the pH 0.1 of a pH).   Immediately after breakfast rinse the mouth thoroughly and take numerous sips of water to remove any remnants of food. Then give third sample.   Samples were then obtained every 15 minutes for an hour, whenever possible, then every hour until

luncheon. After luncheon the same procedure was followed as after breakfast, samples being collected thereafter every hour.

One individual followed this procedure daily throughout the month of July. At the moment of arising from bed the saliva showed a much more acid reaction than at any other time throughout the day. In connection with this point attention should be drawn to an article by Higgins in 1914 in regard to the tension of alveolar carbon dioxide in the same individual assuming different postures. He brought out the fact that the alveolar carbon dioxide was lowest in the erect posture and gradually increased the more relaxed the position, being highest when the subject lay on his back or side, but lower when he assumed the Trendelenburg position. He therefore concluded that the more relaxed the posture the higher the alveolar carbon dioxide.

The second sample taken just before breakfast was in our experience always much more alkaline, though in some cases the interval of time was not over 10 minutes. For several days the mouth was kept tightly closed between the collection of the first two samples and dressing was accomplished slowly and with as little exertion as possible. The results showed a rise in pH, though less marked than usual.

The sample taken immediately after breakfast invariably showed pH 7.0 or over, giving a sharp rise to our curve (see Fig. 1). A few experiments were made on samples taken every 5 or 10 minutes of the hour directly following a meal. The lowest point was usually reached at the end of half an hour, though in some cases the depression continued for $\frac{3}{4}$ of an hour. In every instance where the samples were taken at short intervals the pH was found to fall below that obtained just before breakfast. Thereafter throughout the morning there was a gradual rise, notably in the first 2 hours. If, however, something was eaten during the morning, the characteristic rise again occurred, followed by a fall to a point lower than that found before the consumption of food (see Table II). The rise that followed did not reach as high a level as when food had not been taken. Immediately after luncheon the same sharp rise and fall in pH occurred as after breakfast. Throughout the afternoon the rise was often less marked and seemed to be dependent on how strenuous the day had been. Rarely did we find even the slightest fall in pH, but frequently the rise was not more than 0.1 of a pH.

For a number of days the experiments were carried through the evening. After dinner there was the same peak as after other meals. In one individual we constantly obtained a slight fall after 10 o'clock with only a slight preliminary rise. In two other cases we obtained the usual pH of 6.9. Owing to the limited number of experiments during this period of the day we have indicated them by a dotted line. On one occasion a very strenuous day was spent. In the morning the usual work was done, followed by a very light luncheon and a 70 mile drive. The afternoon was spent in reading as rapidly as possible, the return trip being made after dinner. The saliva was tested at 10 o'clock at the close of the day's work and was found to be more acid than
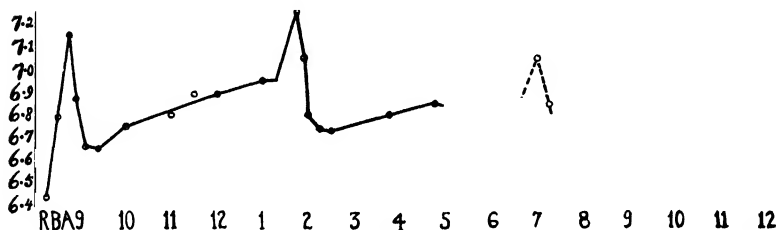
R indicates rising; B indicates before breakfast; A indicates after breakfast.

FIG. 1. Composite daily curve averaged from all figures. The dotted portion of the curve indicates that a limited number of experiments was performed during this period.

ever before in this individual. This same individual gave us samples on an average of twice a week for the past 6 months. These figures showed a remarkable constancy in relation to the time of meals, and in every case there was a fall in pH, lasting $\frac{1}{2}$ to $\frac{3}{4}$ of an hour, to a point below that found before the meal, so that we agree with Starr in stating that there is a slight fall in pH after meals.

These figures together with those obtained from making a daily curve on twelve other individuals, as well as those determined on numerous half days from many more, have been used to form a characteristic curve for the pH of saliva of normal individuals (Fig. 1).

In every case examined, though the figures may have varied 0.1

TABLE I.

	pH									
Before mastication.........	7.0	6.8	6.7	6.8	6.7	6.8	6.7	6.75	6.7	6.8
After "	7.25	7.2	7.1	7.1	7.1	7.2	7.15	7.15	7.15	7.2
10 min.	7.05	7.0	6.8	6.8	6.85	6.9	6.8	6.8	6.8	7.0
20 "					6.8	6.8	6.75	6.8	6.75	6.8
30 "	7.05	6.8	6.75		6.8	6.8	6.8	6.8	6.75	6.8
45 "			6.7	6.8	6.75	6.8	6.75	7.2*	6.75	6.85
60 "		6.85	6.7		6.8	6.8	6.75	7.0	6.75	6.85

* A single walnut was eaten.

TABLE II.

Subject Nos..	1	2	3	4	5	6	7	8	9	10	11	12	Average.
	pH												
Rising....	6.45	6.4	6.6	6.5	6.2	6.5	6.5	7.05*	6.55	6.0	6.6	6.5	6.44
Before breakfast.....	6.8	6.7	6.95	6.8	6.8	6.95	6.8	7.15	6.8	6.9	6.9	6.85	6.86
After breakfast.....	7.2	7.2	7.25	7.0			7.0	7.3			7.2	7.2	7.17
15 min....	7.0	6.75	6.95			6.7	6.7	6.7			6.75	6.8	6.79
30 " ...	6.95	6.7		6.6	6.0	6.5	6.55	6.75	6.8	6.7		6.75	6.63
45 " ...	6.65	6.7					6.65				6.65	6.7	6.67
1 hr......	6.7	6.7	7.0		6.5	6.7	6.65	6.75		6.75		6.7	6.72
2 hrs. ...	6.8	6.8	7.0	6.7	6.6	6.85	6.8	6.8	6.9		7.0	6.9	6.83
3 " ...	6.9	6.8	7.1		6.8	6.9	6.8	6.85	7.0	7.0	7.1		6.92
4 " ...	7.2† 6.65†	6.85		6.85	6.85	7.0	6.8	6.85	7.0	7.05	7.1	6.95	6.93
After luncheon...	7.2	7.2	7.4	7.1			7.1		7.15		7.3	7.2	7.21
15 min....	6.8	6.75	7.0		6.6		6.8	6.9	6.55	6.95†	6.8	6.75	6.79
30 " ...	6.7	6.7	6.9	6.75		6.55	6.65	6.85	6.4			6.8	6.7
45 " ...		6.7					6.5	6.85			6.8	6.6	6.69
1 hr. ...	6.75		7.0	6.75	6.7	6.7	6.65			6.85	6.9	6.8	6.79
2 hrs. ...	6.8	7.1	7.0			6.85	6.75	6.9	6.7		7.0	6.8	6.88
3 " ...	6.85	7.1				6.9	6.8	6.95	6.8	6.95	7.2	6.85	6.92

* Individual moved about before giving this sample.

† Irregularity due to something having been eaten other than at meal times.

or 0.2 of a pH, the same general curve was followed, unless of course a midmorning meal was consumed or candy or ice cream taken during the afternoon, when we again obtained a marked rise and fall of the pH (Table II).

The three peaks in our curve were so brief that we decided to investigate them a little further. Numerous authors had reported that chewing paraffin or pure rubber raised the pH of saliva. In order to avoid the possibility of contaminating the saliva with substances used for mastication we exercised our jaws without chewing any foreign substance. The result was a rise in pH simi-
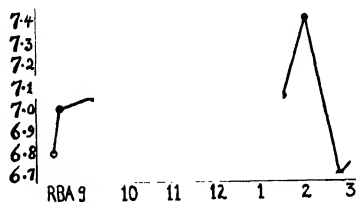
FIG. 2. Daily curve when no breakfast was taken.
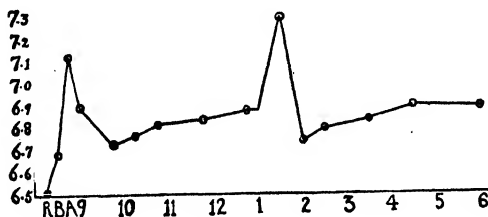
FIG. 3. Daily curve of one individual for 15 consecutive days.

lar to that which we had noted after meals. On testing the pH values for the following hour we found the familiar fall in pH, but, in contrast to the results obtained after meals, the lowest point of depression never fell below that obtained before mastication. Figures for mastication without any stimulant may be found in Table I, while figures for the average daily curve of twelve individuals may be found in Table II.

In connection with this question of mastication we obtained a daily curve on one individual who for breakfast took a cup of coffee requiring no mastication, and for luncheon a dish of soup,

two crackers, and a banana. His curve showed no rise and fall after breakfast and remained constant throughout the morning, except for a slight increase of 0.05 of a pH. After luncheon, however, the characteristic change noted in our general curve again appeared (Fig. 2). We, therefore, suggest that the sharp rise in our curve after meals may be due to mastication.

For comparison we have included a curve of figures obtained from one individual over a period of 15 consecutive days (Fig. 3).

We might readily have reached the same conclusions as Bloomfield and Huck (1920), namely that there was no relation between the ingestion of food and the reaction of saliva, had we not kept the time constant. Had we taken our first sample 30 minutes after eating, we would have obtained many results similar to theirs. On the other hand, if sometimes we had taken the pH within 10 minutes, we should have found a rise in pH, as is seen in one or two cases in their tables.

CONCLUSIONS.

From these experiments we conclude that the salivary pH varies in a very definite way throughout the day, with a tendency to fall after meals to a point slightly lower than that occurring just before meals, and between meals under normal conditions to rise and approach neutrality unless food is consumed. Our maximum range was pH 6.0 to 7.4. If, however, we were to discard the figures obtained on rising and those taken immediately after a meal, our range would be somewhat narrower, or pH 6.5 to 7.1, which is identical with that of D'Alise (1921, 1926), and, for all practical purposes, with that given by Carlson and McKinstry (1924).

Toward the end of our work we were able to check the colorimetric method electrometrically due to the kindness of Dr. Edwin J. Cohn and Dr. F. F. Heyrothe of the Department of Physiology of Harvard Medical School. Dr. Heyrothe made a number of determinations upon their potentiometer, from which we found that there existed a more or less constant difference of 0.2 in the reading, so that our curve if made from figures obtained by this method would be approximately 0.2 higher at every point, our range then being pH 6.2 to 7.6, or, using the narrower range, pH 6.7 to 7.3.

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ZINC AND NORMAL NUTRITION.*

By REBECCA B. HUBBELL and LAFAYETTE B. MENDEL.

(From the Laboratory of Physiological Chemistry, Yale University, New Haven.)

Recent writers on the biochemistry of zinc have stressed two aspects, the hygienic and the physiological. Various workers in the Department of Physiology, Harvard School of Public Health, have been conducting investigations primarily on the hygienic aspects of the question and have found (1, 2) that the addition of comparatively large amounts of zinc to a normal diet had no apparent ill effect on the well being of cats, rats, and dogs. The zinc, in amounts ranging from 175 to 1000 mg. of zinc oxide per day was administered to cats and dogs over periods of time ranging from 3 to 53 weeks. In the case of rats from 2 to 38 mg. of zinc per day, given as the oxide or a zinc salt, had "no significant effect upon the health of the parents, upon fertility, nor upon the health and early growth of the offspring." It was also noted that the excretion by urine and feces fluctuated with the intake. These investigators found that under natural conditions for the rat, at least, there is no significant variation in the zinc content with age, the amount at birth and at old age being almost identical: 0.038 and 0.040 mg. per gm. of rat; while Fairhall (3) writing from the same laboratory, reported rapid storage of zinc in the young growing rat. The relatively low toxicity of zinc has been more recently confirmed from another laboratory (Heller and Burke (4)) so that it seems fairly clear at the present writing that the addition of what may be regarded as excessive amounts of the metal to an ordinary diet is not obviously harmful. It is probable that many of the cases of so called zinc poisoning which have appeared in the

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* The experimental data in this paper are taken from the dissertation submitted by Rebecca B. Hubbell in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1927.

literature can be attributed to a contamination by other metals, such as arsenic, lead, and mercury, which are known to be definitely toxic. It is interesting, as Fairhall has remarked, to note that zinc is a regular constituent of the animal body, while these other elements are not, with the possible exception of arsenic, which seems to be present in very minute quantities.

If then, zinc is not outspokenly toxic, and is a constant constituent of the animal body, what part does it play in nutrition? It has been the claim of some that the metal in small amounts—a fraction of a mg. a day— is an essential in the growth of animals and attempts have been made to show that this is true. The first of these was reported by Bertrand and Benzon (5) who attempted to grow white mice on zinc-free diets to which very small amounts of the metal had been added. The food mixture as fed was composed of highly purified substances to simulate the composition of the wheat kernel, since the writers stated that mice grow well on wheat and water. But the mixture contained none of the recognized sources of vitamins and, as might be expected, growth ceased. The effect of the added zinc was judged by the length of the survival period, since the animals which had received some zinc lived somewhat longer than did those on food which was free from zinc. Only twenty animals in all were used; and the fact that the dietary conditions were such that there could be no growth makes it difficult to compare the results with those of other growth experiments. Another worker (McHargue (6)) made no attempt to remove the contaminating zinc from a food mixture of corn-starch, commercial casein, cane sugar, lard, and salt mixture, but simply added small amounts of a zinc salt to the rest of the food, which was lacking in specified vitamin-bearing material. His report is based on work with rats and he concluded that the addition of zinc to make the moisture-free ration contain about 25 parts per million had a favorable effect on the growth of the animals. These two series of experiments represent the first attempts to show that zinc is an essential in the growth of an animal. The investigations differ radically in that Bertrand and Benzon took great care first to remove all traces of zinc from the constituents of their food mixture and then added a very small known portion of zinc as the sulfate. On the other hand, McHargue made no attempt to remove the metal from the food and

that which was added was superimposed not only on the zinc already present, but also upon other mineral constituents which must have been removed in the rigorous treatment given the food components in the experiments of Bertrand and Benzon.

The present investigation grew out of an interest in the report of Bertrand and Benzon, since it was believed that in order to get a worth while estimate of the effect of zinc on growth, the diet must be so constructed as to contain the factors known to be indispensable. At first an atempt was made to prepare a diet adequate for growth and absolutely free from zinc. Since this proved to be impossible it was thought best to use as a basal food mixture one which was very poor in zinc and then add thereto amounts of zinc to make the quantity of the metal given comparable with that used by Bertrand and Benzon.

The work has thus been divided into two parts. The first was an examination of various substances of plant and animal origin with reference to their zinc content. This was done partly to confirm and extend the work of others and also as a necessary preliminary to the feeding experiments which were to follow. The survey corroborated the previously reported wide-spread presence of zinc and also showed again that there is no great variation in the amount of zinc present in a given type of material from different sources. The second part of the investigation consisted of feeding experiments with white mice. Studies were made of the growth and zinc content of the animals on various diets. A standard basal diet was used as a contròl and this diet was varied, first, by the greatest possible exclusion of zinc, thus forming a so called zinc-low diet. Known amounts of zinc were then added to this zinc-low diet in two groups. In one group 0.02 mg. of zinc per mouse per day was added and in the other group 0.04 mg. per mouse per day. In each case the zinc was added as a solution of the sulfate.

Methods of Analysis.

A survey of the literature shows that for the determination of minute amounts of zinc few methods are at all reliable. Since the zinc present in living material is probably to some extent at least in organic combination, it is necessary first thoroughly to ash the material to be analyzed. In this investigation two methods

of destroying the organic matter have been used.   The first was the usual "wet ashing" with sulfuric and nitric acids.   The procedure which seemed most satisfactory was the following.

The material was first dried in the oven at 110°C., and broken into small pieces, placed in a 700 cc. Pyrex Florence flask, and concentrated sulfuric acid added.   The amount of acid used varied with the weight and type of organic material to be destroyed.   The flask was then allowed to stand for several days until the material was thoroughly disintegrated, after which it was heated on the steam bath for 6 to 10 hours.   At the end of this time all lumps had disappeared and the entire mass was semifluid.   Concentrated nitric acid was then added in small amounts, about 2 cc. at a time, until the first violent action had ceased.   The addition of the nitric acid was continued until no more fumes were given off.   The flask was then transferred to an electric hot plate and heated gently until the nitric acid had decomposed and the sulfuric acid fumes appeared.   The flask was then cooled slightly, a small amount of nitric acid again added, and the flask heated on the hot plate until the sulfuric acid fumes again appeared.   This addition of nitric acid and subsequent heating was repeated until the mass had become corlorless.   The heating was then continued for 5 to 6 hours until much of the excess sulfuric acid had been driven off.   The digest, after being cooled, was diluted with a small amount of water, partly neutralized with ammonia (1:1), transferred to a 400 cc. Pyrex beaker, and the neutralization completed, methyl red being used as an indicator.   After the addition of a small amount of sodium acetate and acetic acid until the reaction was acid, the solution was heated to a temperature just below the boiling point and the zinc precipitated by a lively stream of hydrogen sulfide.   The precipitate was allowed to stand overnight, filtered off in the morning upon a No. 40 Whatman filter paper (9 cm.), and washed with hydrogen sulfide water.   The precipitate was treated on the filter paper with 1:9 hot hydrochloric acid to dissolve the zinc sulfide.   The filtrate was freed from iron (derived partly from reagents) by heating with bromine water to oxidize the iron to the ferric state, adding dilute ammonia drop by drop until the iron was precipitated (solution faintly alkaline), and the zinc salt, still in solution, was separated from the precipitate by filtration.   This solution was used for the final determination of the zinc.

The other method of ashing was the usual dry process.   An electric furnace was used, and the material heated at a maximum temperature of 450°C.   At this temperature there is no appreciable loss of zinc by volatilization (Thompson (7)).   The material was first dried, as in the case of the moist ashing, then heated in a porcelain evaporating dish on a hot plate until it was completely charred, and was then transferred to the electric furnace and the temperature gradually raised to the maximum of 450°C.   It was found best to introduce the charred mass into a cool furnace, to avoid the sudden flaming which sometimes occurred if the material had been incompletely charred.   In 3 or 4 hours the ashing was complete, with the

exception of occasional small particles. A few drops of nitric acid, added at this point, hastened the process, and heating in the furnace for an hour more was enough for complete ashing in most cases. The ash was then extracted with 1:1 hydrochloric acid and the washings were filtered. The residue on the filter paper was examined carefully for any unashed material. If such was present, the filter paper was dried, paper and ash heated in the furnace again until ashing was complete, and the extraction with hydrochloric acid was repeated. This second ashing, was, however, rarely necessary if nitric acid had been added just before the final heating in the furnace. The acid filtrate was treated with bromine water to oxidize iron, and bromine in excess removed by boiling, and the solution made neutral by the addition of ammonium acetate. In the analysis of animals it was necessary to remove phosphate at this point. This was done by dissolving the precipitate on the filter paper in hydrochloric acid and reprecipitating the phosphate, to get rid of any zinc which might have been adsorbed with the iron phosphate. The acid solution was then treated with ammonium acetate and added to the original. The combined neutral solutions were treated with hydrogen sulfide, the sulfides filtered off, and washed with hydrogen sulfide water. The zinc sulfide was then dissolved out by 1:9 hydrochloric acid, the solution made neutral by ammonia, and this solution used for the final determination of zinc.

For the actual estimation of the zinc two methods have been used. The first was the usual titration with potassium ferrocyanide, uranium acetate being used as an outside indicator. This has been found satisfactory if carefully controlled conditions for carrying out the titration were adhered to. As a rule the volume of the zinc solution used was 50 cc.; in this volume, with a 1 per cent uranium acetate solution as indicator, it was possible to estimate accurately amounts of zinc ranging upward from 0.1 mg. per 10 gm. sample, though this method was not generally used when the expected amount fell below 0.5 mg. per sample. As it was not always advisable to have the volume exactly 50 cc., a preliminary determination was made of titration conditions for volumes ranging from 25 to 75 cc. The amount of potassium ferrocyanide needed to get a reaction with the indicator for different volumes was found. In each determination, the actual volume was measured and the proper correction made for the amount of potassium ferrocyanide used in excess of that reacting with the zinc.

The other method used also involved titration by means of potassium ferrocyanide, but the end-point was obtained by the potentiometric method of Van Name and Fenwick (8). This was found to be very satisfactory for large or small amounts of zinc, though in the determination of amounts below 1 mg. per sample it was found best to add a definite amount of zinc chloride solution to the unknown before starting the titration, and so get the amount of zinc in the unknown by difference. While the electrometric titration of zinc has been used to some extent in various investigations, very little mention is made of the method in connection with the determination of the metal in biologic material. The method as outlined by Van Name and Fenwick is briefly as follows: The electrode system "consisted

of two identical pure platinum electrodes between which a very low polarizing current was allowed to flow continuously. The latter was obtained by applying a constant potential of from 0.20 to 1.00 volt and interposing a fixed resistance of 20,000 ohms in series with the two electrodes. Polarized electrodes have the great practical advantage of giving more definite and reproducible potentials than those given by the unpolarized electrodes." With these polarized platinum electrodes was used a special form of silver-silver chloride electrode of the type described by Willard and Fenwick (9). By plotting millivolts against cc. of ferrocyanide used, the end-point was determined, as the point of greatest drop in potential. In all titrations, micro burettes, graduated to 0.02 cc., were used.

The potentiometric method was used where small amounts of zinc were expected and was found to be very satisfactory. The accuracy of the method was tested by adding varying amounts of zinc to purified cornstarch and recovering the metal as indicated. Amounts ranging from 0.05 mg. per 10 gm. sample of starch upward were used and it was found possible to estimate such amounts with an error of 5 to 8 per cent.

Preliminary Analyses.

Analyses of animal and vegetable products from various sources were made and these verified to some extent the results which had been obtained by other workers from time to time (10, 11). These and the analyses of materials to be used in the diets for the second phase of the study are reported in Table I.

In planning a zinc-low diet, the food mixture described by Beard (12) as the optimum for mice was used as a basis. This consists of:

	per cent
Casein	31
Starch	38
Crisco	21
Cod liver oil	3
Salt mixture	7

100 mg. of yeast powder per day.

The zinc content of this mixture as usually made up in the laboratory was found to be 106 mg. per kilo. This provides a daily intake of 0.318 mg. of zinc per mouse per day, on the basis of the average daily food intake of 3 gm. as ascertained by Beard. Early in the study, attempts were made to reduce the amount by substituting other food ingredients which were lower in zinc content. An analysis of egg white showed that its zinc content was low and further purification removed that originally present.

TABLE I.

Zinc Content of Various Food Products.

Material.	No. of samples.	Weight of sample.	Zinc content per kilo.
		gm.	mg.
Peas I*......	3	100	51.7
" II......	3	100	46.2
" III.....	2	100	40.1
Carrots I....	2	100	43.1
" II..	3	100	34.4
" III.	2	100	29.5
Beets I......	3	100	38.7
" II.....	2	100	17.8
Cabbage I..	2	100	12.9
" II.	2	100	17.8
Milk I.......	2	100	29.4
II..	2	100	32.1
III.	2	100	25.5
IV..	2	100	30.0
Egg yolk I....	2	100	42.9
" " II..	2	100	38.1
" " III.	2	100	40.1
Oysters I..	2	100	286.5
" II.	2	100	412.2
Clams I....	2	100	15.2
" II...	2	100	22.1
Lettuce I..	2	100	5.1
II.	2	100	8.9
Corn-starch.	5	10.0	21.0
" (washed).	5	25.0	00.0
Casein (commercial)..	4	10.0	309.0
(purified).	5	25.0	4.0
Egg white (commercial).	2	10.0	21.0
(purified).	2	20.0	00.0
Crisco.	3	10.0	10.0
Yeast powder.	5	10.0	14.0
Cod liver oil..	3	10.0	9.0
Salt mixture...	3	10.0	00.0

* Roman numerals indicate samples from different sources.

Beard had reported that mice grew almost as well on egg white as they did on casein, hence the substitution seemed justified. Further work showed, however, that commercial casein could be

quite easily freed from the major portion of zinc; consequently the egg white was abandoned in favor of the preferable protein. The method of purification for the casein was as follows:

200 gm. of commercial casein were suspended in about 3 liters of water which was specially distilled from glass since zinc was found to be present in the ordinary distilled water of the laboratory. The reaction of this water suspension was distinctly acid. A few drops of 1:1 ammonia added to the suspension sufficiently reduced the acidity, so that a deep yellow liquor was washed out. The liquid was decanted off through fine cheese-cloth and the washing was repeated five or six times, the suspension being stirred for 10 minutes each time with an electric stirrer, until no more yellow color was obtained in the wash water. An analysis of the fluid showed the presence of a reducing sugar and it is assumed that the color was due to caramelized lactose. Portions of the washed casein were then dissolved with constant stirring in dilute ammonia, care being taken to avoid excess of ammonia and the consequent denaturization of the casein. The simplest way of doing this seemed to be to keep a small amount of the casein undissolved and to stir until the alkaline reaction had disappeared. This dilute neutral solution was allowed to stand for several hours until particles of dirt had settled. The solution was poured off through two thicknesses of fine cheese-cloth and stirred vigorously again, dilute acetic acid being added slowly until the reaction was well on the acid side of the isoelectric point, in order to insure complete solution of the phosphates. When the precipitation had been done with care and in sufficient dilution, the precipitated casein gathered at the top, upon continued stirring, partly in the froth and partly in somewhat larger particles. The casein was easily separated from the liquid, washed by suspension, with repeated stirring and filtering, until neutral. The solution of the casein and subsequent reprecipitation with acetic acid were repeated. It was found that if the solutions were very dilute and the precipitation had been done very slowly, one reprecipitation was enough. After the last reprecipitation and washing the casein was dried by the use of alcohol and ether in the usual way. A product was obtained which was very light in color. Analysis of the casein showed a nitrogen content of 14.7 per cent as compared with 12.6 per cent for the crude, and the zinc content was 0.004 mg. per gm. instead of 0.309 mg. per gm. for the crude.

Bertrand used potato starch, because the grains are large and settle easily, thus facilitating purification by washing. It was found, however, that a good brand of commercial corn-starch had a very low zinc content and could be washed completely free of zinc more easily than the potato starch could be made in quantity. The method of washing was briefly as follows:

The starch was suspended in distilled water to which a few drops of hydrochloric acid had been added, stirred for 10 minutes, and then allowed to

settle for about a half hour, during which time considerable dirt settled out. Analysis of this residue showed that its zinc content was high, so that it is probable that part of the zinc present in the starch was there in the dust. The upper layers of the starch suspension were separated from the lower by decantation, again suspended in acidulated water, stirred for 10 minutes, and the suspension allowed to stand overnight. The clear supernatant liquid was poured off and the washing repeated three times more. The cake of purified starch was allowed to dry in the air for 2 days and then in the oven at a low temperature. This product gave a very low ash and only one sample out of many showed any zinc.

Since the commercial fat, Crisco, contained a small amount of zinc and the problem of purification presented unusual difficulties, lard was used instead and was prepared directly from the leaf fat as follows:

The fat was carefully freed by dissection from all blood vessels and from connective tissue, as far as possible. It was then heated on the steam bath until the fat was just melted, and strained through two layers of fine cheese-cloth. To purify further, the lard was heated to its melting point in distilled water, the whole shaken thoroughly, and the lard separated from the water in a separatory funnel. The lard so obtained contained a small amount of water which was removed by heating again on the steam bath just to the melting point and then pouring off the upper layers of the water-free fat. A product was thus obtained which was zinc-free and practically water-free.

It was thus possible to obtain almost free from zinc the three substances needed in large amounts in the food mixtures. The Osborne and Mendel (10) salt mixture was used. The zinc content of this as prepared from selected reagents was found to be so low as to be practically negligible.

The problem of choosing suitable vitamin-bearing materials was only partly solved. As a source of the antixerophthalmic and antirachitic factors a concentrate from cod liver oil was used.[1] This material is reported to contain in 1 gm. the unsaponifiable portion of 100 gm. of cod liver oil, so that the food as made up required only 30 mg. of the concentrate per 100 gm. of food, or 0.9 mg. per mouse per day, using the value for food intake indicated before.

Many sources of vitamin B were tested and zinc was found

[1] This was supplied through the courtesy of Dr. H. E. Dubin of the Metz Laboratories. No analysis was made of the product by the writers since Dr. Dubin gave assurance of its freedom from zinc.

present in all, so that it seemed best in the end to use material of known potency in the growth of mice, even though it contained a small amount of zinc. As Beard had found that mice failed to grow above a body weight of 18 gm. with the use of purified diets and the Harris vitamin concentrate as a source of vitamin B, yeast powder with a zinc content of 0.014 mg. per gm. or 0.0014 mg. per mouse per day was used. The so called zinc-low diet was then essentially that which Beard had found satisfactory for mice, the only changes being the substitution of lard for the hydrogenated fat and of cod liver oil concentrate for the cod liver oil itself.

In feeding several problems arose. Mice tend to scatter food much more than do rats. Beard had partly overcome this difficulty by placing the food in an elbow of metal from which only a small amount of food could be removed at a time. This method, however, did not seem practicable for us, since it made the problem of keeping track of food and zinc intake more difficult. Preliminary experiments with the animals showed that growth occurred at a normal rate if the food was first cooked enough to form a thick paste. This was done by adding 3 gm. of water for each gm. of food, and heating the mixture for about 10 minutes on the steam bath. This heating, of course, did not appreciably affect the vitamin-bearing materials and made a more homogeneous preparation than mere mixing usually does. There was very little scattering of this paste food compared with the excessive scattering when the food was fed dry. The steaming also gave an easy way of incorporating the zinc in the diet, for in cases where the element was to be included, a measured amount of zinc sulfate solution was added to the food before it was cooked. The food mixtures as finally used were:

Diet I, Standard Diet of Beard.	*per cent*	*Diet II, So Called Zinc-Low Diet.*	*per cent*
Casein......................	31	Zinc-low casein............	31
Corn-starch.................	38	Zinc-free starch...........	40
Crisco......................	21	" lard..............	22
Cod liver oil...............	3	Salt mixture..............	7
Salt mixture...............	7	Cod liver oil concentrate.	0.03
100 mg. of yeast powder per mouse per day.		100 mg. of yeast powder per mouse per day.	
		This mixture furnished 0.005 mg. of zinc per mouse per day.	

Diet III.—This was the same as Diet II with the addition of a solution of zinc sulfate sufficient to make the zinc content of the diet 0.02 mg. per mouse per day.

Diet IV.—This was the same as Diet II with the addition of a solution of zinc sulfate sufficient to make the zinc content of the diet 0.04 mg. per mouse per day.

Cages and Care of Animals.

The cages employed were selected to be suitable for feeding trials by excluding metal. During the course of the experiment
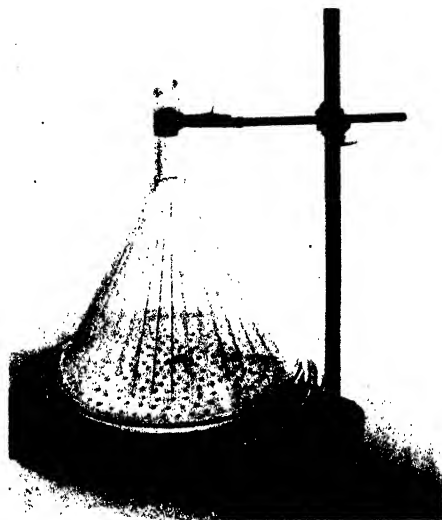
Fig. 1. Photograph of porcelain-glass cage.

several types of porcelain-glass cages were used. Only one will be described here, since it proved to be simple in construction, easy to clean, and apparently satisfactory for the comfort of the animal.

As the photograph shows (Fig. 1), the cage consisted of a glass pie plate, a porcelain desiccator plate, and a $9\frac{1}{2}$ inch short stem glass funnel. The desiccator plate was supported an inch from the bottom of the pie plate by means of a porcelain food cup, inverted and placed beneath the center of the desiccator plate. The

plate, so elevated, served to some extent as a false bottom for the cage, allowing some of the feces and urine to fall through. Over the desiccator plate was placed the glass funnel, held in place by a clamp. The temperature of the interior of this cage was tested over periods of 3 or 4 days throughout the investigation and found to vary but slightly from that of the room outside. The only difference noted was that there was less fluctuation in temperature than in the room as a whole. Adequate circulation of air was provided by the holes in the porcelain plate, and there was in but few cases at most any evidence that ventilation was not complete. In order to make sure that the cage as devised was suitable for the growth of mice on a tested standard diet, as far as general conditions of atmosphere and temperature were concerned, a comparison was made between mice in the usual wire cages and in the metal-free ones. The growth curves showed that there was no variation other than that usually shown by different experimental animals in the same group.

In the present investigation the cages were used for mice, but with a view to future work, they were also tested with rats and were found to be entirely satisfactory.

The cages were washed frequently, the whole cage once a week and the desiccator plate three times a week or more often, if at any time there was indication that small amounts of discarded food, feces, or urine had not passed through the holes. The glass funnel was found to be very satisfactory in preventing contamination of the cage by dust from the air. Analysis of the air of the animal room and of various laboratories had shown a definite zinc content, particularly in a room filled with the usual wire cages. To avoid any possible contamination of this sort, the porcelain-glass cages were kept, during the experiment, in a special room, where the atmosphere was comparatively free from dust and where there were no metal cages for other animals.

The mice used were bred in the laboratory, and only those in good condition and reasonably uniform in weight were used for the experiments. As far as possible comparisons were made within litters. From a litter of six animals, for example, two were fed on the standard diet (Diet I), two on the zinc-low diet (Diet II), and two on the zinc-low diet to which had been added a definite amount of zinc (Diet III or Diet IV). In some litters of six,

instead of two animals being used for controls, one was killed and the zinc content determined and the other used as a control.   In this way it was possible to check throughout the entire experiment both the presumable zinc content of the animals at the start and the growth on the control diet.   In neither case were there significant variations from the values obtained in the preliminary work.   At no time were animals put on Diets II, III, or IV without at least one animal for comparison on Diet I.

Since it was not considered necessary to measure food intake in all phases of this work, in a few cases two animals of the same sex were kept in the same cage, though in most cases, only one animal was in a cage, according to the standard procedure.   In the instances where more than one animal occupied a cage, great care was taken to note any differences as compared with animals kept separately.   In no case was there any evidence that an animal was unfavorably affected by the presence of another.   It was considered necessary to measure food intake only of animals receiving a definite daily ration of zinc in addition to the zinc-low diet.   In the other cases enough food was given to satisfy the needs of the animals, about 3 gm. per day, as determined by Beard.   There was no evidence at any time of underfeeding, for in most cases there was a small amount of food left untouched.   In the case of animals receiving zinc, however, a close watch was kept, and from time to time the food which was not eaten was collected, dried, and the zinc content determined, in order to find out how much zinc the animals were really getting.   From the data obtained the amount of zinc sulfate actually added was adjusted, so that the animal should receive as nearly as possible the amount of zinc desired, 0.02 or 0.04 mg. per mouse per day.

DISCUSSION OF RESULTS.

Data were obtained on the rate of growth and zinc content of both male and female mice on the four diets.   See Table II and Chart I.   A study of the curves shows in general a slight retardation of growth on the zinc-low diets, with a slight favorable effect when the smaller of the two amounts of zinc was added to the zinc-low diet.   When a larger amount of the metal was added, 0.04 mg. per mouse per day, there was not so marked an increase in growth rate.   A statistical study of growth, taking weights at

20, 45, and 70 days after the animals had reached 10 gm. of body weight shows more clearly the *slight* stimulating effect of the addition of small amounts of zinc. This is especially true in the growth of female mice. The following comparisons were made, for both males and females.

1. Growth on Diet I compared with that on Diet II.
2.   "   "   " II   "   "   "   "   " III.
3.   "   "   " II   "   "   "   "   " IV.
4.   "   "   " I   "   "   "   "   " III.
5.   "   "   " I   "   "   "   "   " IV.

TABLE II.

Average Weights of Mice at Three Different Intervals in Their Growth.

Diet No.	No. of animals.	Weights in gm. at three different age intervals after mice had reached 10 gm. of body weight.		
		20 days.	45 days.	70 days.
Females.				
I	11	15.8	19.1	21.6
II	15	15.5	17.6	18.8
III	14	17.1	19.9	21.1
IV	9	16.3	18.0	19.4
Males.				
I	9	20.5	23.2	25.8
II	12	17.0	20.2	23.3
III	10	18.8	23.4	24.3
IV	8	18.5	21.7	22.9

In this way an idea was obtained as to whether (1) there was retardation in growth with the removal of zinc from the diet; (2) whether the addition of small amounts of the metal stimulated growth in any way, and (3) if there was found to be stimulation in growth, whether the addition of zinc made the diet as nearly adequate as the standard. The results are expressed in Table III by the significance ratio $\dfrac{D}{PEd}$ and are to be interpreted as follows: For female mice when the growth on Diet I is compared with that on Diet II for the ages indicated it was found that the ratios were: for 20 days, -1.04; for 45 days, -3.8; and for 70 days, -7.4,

showing that in the first period the change in rate of growth on the zinc-low diet was not great enough to be significant, while in the other two periods there was a significant retardation in growth when the animals were on a food mixture low in zinc. This is indicated by the negative values −3.8 and −7.4. A study of Table III shows the following.

1. There was a retardation in growth when the animals were fed on a zinc-low diet.

2. When 0.02 mg. of zinc per mouse per day was added to the zinc-low diet, there was a stimulation in growth as compared with that on the zinc-low diet. This was not quite so marked with the males as with the females.
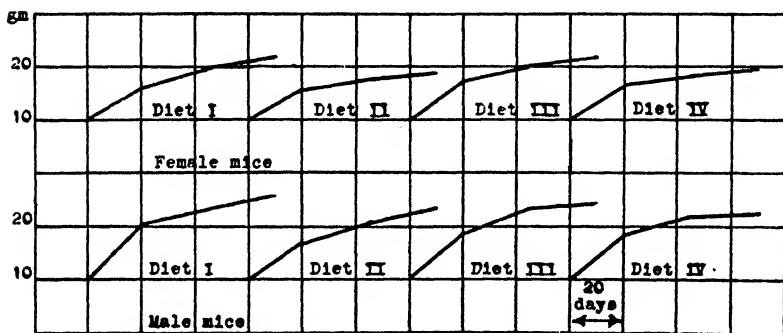
CHART I. Curves showing slight variations in growth on different diets. Diet I, control; Diet II, zinc-low; Diet III, zinc-low plus 0.02 mg. of zinc per mouse per day; Diet IV, zinc-low plus 0.04 mg. of zinc per mouse per day.

3. When the larger amount of zinc, 0.04 mg. per mouse per day, was added, there was not the same stimulating effect on growth which was noticed when the metal was given at the lower level. The behavior of the animals was more irregular than in any of the other groups. In some cases there seemed to be a slight stimulation while in others there was a distinct retardation in growth.

4. When comparison was made between the animals on Diet III and the control, Diet I, it was found that on the whole the addition of zinc, while it caused some stimulation and made the diet more nearly adequate than that from which most of the metal had been removed, did not alone restore the food mixture to the level of the control.

On the whole it would seem that the addition of small amounts of zinc, approximately 0.02 mg. per mouse per day, to a diet containing a very minute amount of the metal had a positive effect on the growth of both males and females, but that the stimulating

TABLE III.

Comparison of Growth of Mice on Different Diets by Calculation of the Significance Ratio $\dfrac{D^*}{PEd}$.

Diet No.	$\dfrac{D}{PEd}$		
	20 days.	45 days.	70 days.
Females.			
I –II	−1.04	−3.8	−7.4
II–III	+3.3	+4.9	+7.0
II–IV	+2.35	+0.7	+0.8
I –III	+3.3	+1.5	−2.1
I –IV	+2.4	−3.2	−3.4
Males.			
I –II	−6.8	−6.8	−5.5
II–III	+2.21	+4.6	+1.8
II–IV	+2.6	+2.4	−1.3
I –III	−3.0	+0.1	−3.75
I –IV·	−4.4	−3.5	−9.6

*The calculation of the significance ratio $\dfrac{D}{PEd}$ was made by the use of the following.

Standard deviation $= \sqrt{\text{Average of squares of deviations from the means.}}$

Probable error $= \dfrac{\text{Standard deviation} \times 0.6745}{\sqrt{\text{No. of cases}}}$.

"   of difference $= \sqrt{(PE \text{ mean }_1)^2 + (PE \text{ mean }_2)^2}$.

If D, the difference between two means, is greater than 3 times PEd, the difference is considered significant.

effect was more marked with the females than with the males. It is realized that the figures for Diet IV are necessarily incomplete, because of the smaller number of animals used.   In considering these results it should be borne in mind that at no time during the

experiments did the animals consistently lose weight. In all cases there was active growth and the mice were normal in appearance. The differences noted were between animals all of which were receiving food adequate for growth and any variations between groups can be attributed to the effect of zinc on normal or nearly normal metabolism. In any event, the inclusion of zinc in the proportions used was not in any evident way detrimental to the growth of the animals as a whole.

Determination of Zinc Content of Experimental Animals.

In addition to the observations on the growth of mice on the various diets, an extended examination was made of the zinc content of:

1. Animals on the stock diet at various ages.

2. Animals on the experimental diets. With these animals the determinations were made at the age of 70 days after they had reached 10 gm. of body weight.

A preliminary study was made to determine the relation of zinc content to age in order to detect a possible normal storage of zinc. These figures were also compared with data for the zinc content of the experimental animals to find out whether there was any marked reduction in the zinc content of the body with a diet low in the metal. The animals were killed 24 hours after feeding, by exposure to illuminating gas. The alimentary tract below the empty stomach was removed to eliminate all food residues, and the whole animal dried at 110°C. The desiccated animal was then preserved in a glass bottle for analysis. The same method as that described for the analysis of food materials was used, with the noted adjustments for the additional phosphates of bone.

The preliminary survey included 78 animals ranging in age from 1 day to 300 days on the stock diet. The average zinc content ranged from 0.021 mg. to 0.030 mg. per gm. of mouse, the lower figure being for the animal at birth and the higher for those in old age. It is interesting to note in Table IV that the values for animals at 22 to 70 days, or in the period of active growth, show very little real variation in the zinc content. The slight increase or storage seems to come with old age when the body is not metabolizing so actively.

TABLE IV.

Zinc Content of Stock Animals.

No. of animals.	Age.	Average body weight.	Average zinc content per gm. of mouse.
	days	*gm.*	*mg.*
7 *	1	1.32	0.021
10 ♂	22	9.5	0.027
11 ♀	22	7.8	0.024
10 ♂	35	11.5	0.026
9 ♀	35	9.8	0.024
7 ♂	50	20.5	0.027
8 ♀	50	16.2	0.026
5 ♂	70	23.6	0.028
6 ♀	70	22.1	0.025
4 ♂	175 to	28.5	0.030
5 ♀	300	24.6	0.029

* In the case of animals analyzed at birth the figure 7 refers to *determinations* and not to the number of animals. In a single determination from three to six animals were used.

TABLE V.

Zinc Content of Experimental Animals Determined 70 Days after 10 Gm. of Body Weight Had Been Reached.

Diet No.	No. of animals.	Average body weight.	Average zinc content per gm. of mouse.
		gm.	*mg.*
I	9 ♂	25.8	0.019
	11 ♀	21.6	0.021
II	12 ♂	23.3	0.012
	15 ♀	18.8	0.016
III	10 ♂	24.3	0.021
	14 ♀	21.1	0.032
IV	8 ♂	22.9	0.032
	9 ♀	19.4	0.029

The analyses of animals (Table V) on the experimental diets showed the following.

1. The animals fed on the zinc-low Diet II showed a distinctly lower zinc content than did those on the control Diet I. A decrease from 0.021 to 0.016 mg. per gm. for females and from 0.019 to 0.012 mg. per gm. for males was noted.

2. Animals which had been fed 0.02 mg. of zinc per mouse per day in addition to the small amount (0.005 mg. per mouse per day) in the zinc-low diet, showed a zinc content somewhat above that of the animals which received the smaller amount of the metal.

3. With the feeding of the larger amount of zinc (0.04 mg.) the amount retained was increased for the males, but not for the females.

4. In both groups mentioned in (2) and (3) the zinc content is comparable with that of the control animals and those on the stock diet, though the intake is much smaller. With the control animals the intake was approximately 0.318 mg. of zinc per day and with the animals on the stock diet, approximately 0.224 mg., amounts far in excess of the zinc added in Diets III and IV.

SUMMARY AND CONCLUSIONS.

A study has been made of the zinc content of some common foods, of the effect of the metal on the growth of white mice, and of the relation of the zinc content of mice to their diet. The brief survey served to corroborate the reports of earlier investigators that zinc is quite commonly present in small amounts in foods of both plant and animal origin and that the amount present does not vary widely for a given type of material, even though the samples are taken from quite different regions.

In the growth studies, animals in metal-free cages of glass and porcelain were fed a zinc-low diet, containing in the day's ration an average of only 0.005 mg. of zinc. The effect of the element on growth was then tested by adding to this zinc-low diet enough zinc sulfate to make the total intake either 0.02 or 0.04 mg. of the metal each day. A study of the growth showed that the addition of the smaller amount was attended by growth very nearly normal, while the addition of the larger amount was less effective. The slightly favorable effect of adding 0.02 mg. was more evident with the females than with the males.

Analyses of the animals at a uniform age, 70 days after they had reached 10 gm. of body weight, showed slight variations on the different diets. The animals on the zinc-low diet showed a slight loss in body zinc as compared with the controls, while those which had received additional zinc showed some increase. The increase was not pronounced in any case, so that in an actively growing mouse there does not seem to be storage of zinc to any extent.

While there is some evidence from the work done that the addition of a small amount of zinc may cause a very slight stimulation in growth, it seems probable that the addition of zinc alone to a food mixture low in the metal is not sufficient to make the diet equal to standard. It is possible that any value which zinc may have lies not alone in the presence of the metal itself, but that it may be in some way associated in function with other metals present in small amounts. It is not unlikely, however, that there is some variation in growth with varying amounts of zinc and that the metal is not merely an accidental factor in the nutrition of the mouse.

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ON WALDEN INVERSION.

XI. ON THE OXIDATION OF SECONDARY MERCAPTANS TO CORRESPONDING SULFONIC ACIDS AND ON THE WALDEN INVERSION IN THE SERIES OF SECONDARY CARBINOLS.

By P. A. LEVENE and L. A. MIKESKA.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The observations on the halogenation of optically active secondary alcohols previously reported[1] have now been extended to a larger group of substances. The alcohols thus far employed belong to four groups.

To the first belong the simple aliphatic carbinols. Of these the simplest is methylethyl carbinol. In the other alcohols of this group the ethyl is replaced by a higher homologue, such as propyl, or hexyl, or by a radical containing a secondary carbon atom, such as isobutyl.

The second group of alcohols contains a phenyl group. The simplest member of this series is methylphenyl carbinol. In the other members of the series the methyl group is replaced (1) by a higher homologue such as ethyl, propyl, or butyl, (2) by a radicle containing a tertiary carbon atom, (3) by benzyl, (4) by cyclohexyl.

To the third group belongs methylnaphthyl carbinol.

To the fourth group belong cyclohexylmethyl and cyclohexylethyl carbinols.

An attempt was made to halogenate every alcohol by two reagents, one being thionyl chloride, the other either hydrogen halide or phosphorus pentahalide. In two cases, namely in n-propylmethyl and in cyclohexylmethyl carbinols, the first reagent failed to halogenate. The results of the halogenations are

[1] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, 1925, lxv, 507; 1926, lxx, 355.

TABLE I.

Carbinol. $[M]_D^{20}$ (1)	Halide. $[M]_D^{20}$ (2)	Thiol. $[M]_D^{20}$ (3)	Sulfonic acid.	
			$[M]_D^{20}$ Free acid. (4)	$[M]_D^{20}$ Salt. (5)
Methylhexyl.				
$-9.8°$ (HBr).............	$+25.9°$	$-12.6°$	$+4.9°$	
$-9.8°$ (SOCl₂).............	$+20.6°$			
Methylethyl.				
$+4.94°$ (HI)...............	$-3.2°$	$+15.98°$	$-4.38°$	$-6.29°$
$-3.15°$ (SOCl₂).............	$+1.13°$			
n*-Propylmethyl.				
$+16.3°$ (PCl₅).............	$-13.4°$	$+8.2°$	$-4.87°$	$-5.51°$
Isobutylmethyl.				
$+22.8°$ (HI)...............	$-38.1°$	$+25.0°$	$-11.15°$	$-11.7°$
Benzylphenyl.				
$+36.76°$ (SOCl₂)............	$-23.1°$	$+18.3°$	$-95.4°$	$-159.5°$
$+33.1°$ (PCl₅).............	$-4.6°$			
Methylphenyl.				
$+11.0°$ (SOCl₂)............	$+15.4°$	$-11.0°$		
$+59.9°$ (PCl₃).............	$-6.1°$			
Ethylphenyl.				
$-74.2°$ (SOCl₂)............	$-77.8°$	$+64.8°$	$-3.00°$	
$+47.4°$ (HBr).............	$-3.0°$			
Cyclohexylphenyl.*				
$-38.3°$ (SOCl₂)............	$-62.3°$	$+89.2°$	$+12.18°$	$+11.97°$
$-8.5°$ (PCl₅).............	$+9.9°$			
***n*-Propylphenyl.**				
$+35.1°$ (SOCl₂)............	$+45.3°$	$-66.7°$		
$+40.3°$ (HBr).............	$+9.8°$			
***n*-Butylphenyl.**				
$-21.7°$ (SOCl₂)............	$-31.8°$	$+32.3°$		
$-20.1°$ (PCl₅)	$-9.3°$			
Isopropylphenyl.				
$-20.4°$ (SOCl₂)............	$-27.9°$	$+25.2°$		
$+70.5°$ (HBr).............	$+58.5°$			
Methyl-α-naphthyl.*				
$-60.5°$ (SOCl₂)............	$+69.6°$	$-46.6°$	$-157.8°$	$-146.9°$
$-60.5°$ (PCl₅).............	$+10.3°$			
Cyclohexylmethyl.*				
$-5.0°$ (PCl₅).............	$+5.6°$	$-5.2°$	$+17.47°$	$+16.92°$
Cyclohexylethyl*				
$+13.0°$ (SOCl₂)..	$+2.2°$			
$+12.7°$ (PCl₅).............	$-1.9°$			

* Substance not reported upon previously.

summarized in Table I. All substances marked with an asterisk have not been reported upon previously.

From Table I it is seen that the result of the action of the identical reagent varies, depending upon the character of the radicles contained in the molecule of the carbinol. Thus, (a) in the first group of alcohols halogenation by any one of the reagents results in a change of the direction of rotation. (b) In the phenyl carbinols the result of the halogenation is determined by the second radicle. Three different results are observed in this group. (1) Both reagents bring about a change in direction of rotation as in the first group. This result was observed only in benzylphenyl carbinol. (2) Both reagents halogenate without changing the direction of rotation of the carbinol. This behavior was observed in n-propyl, isopropyl, and n-butylphenyl carbinols. (3) One reagent (thionyl chloride) halogenates without changing the direction of rotation, whereas the other reagent brings about a change in the direction of rotation. The substances reacting in this manner are methyl-, ethyl-, and cyclohexylphenyl carbinols. (c) Methyl-naphthyl carbinol reacts with thionyl chloride and with phosphorus pentachloride to give chlorides rotating in the direction opposite to that of the alcohol—thus resembling in this respect the simple aliphatic secondary carbinols. (d) Cyclohexylmethyl and -ethyl carbinols react with phosphorus pentachloride to give a chloride rotating in the direction opposite to that of the parent alcohol, whereas thionyl chloride with cyclohexylethyl carbinol gives rise to a chloride rotating in the same direction as the parent carbinol.

It is evident that those carbinols which give rise to two enantiomorphous halides must suffer a Walden inversion in one of the two reactions, as was pointed out first by McKenzie[2] regarding methylphenyl carbinol.

In the cases when both reagents act in the same sense, either changing the direction of rotation (as in the aliphatic alcohols, in benzylphenyl and in methylnaphthyl carbinols) or leaving the direction of rotation unchanged (as in propyl-, isopropyl-, and butylphenyl carbinols), it is possible to assume either inversion by both reagents, or no inversion by either reagent.

As stated in previous articles, in order to find an answer to the

[2] McKenzie, A., and Clough, G. W., *J. Chem. Soc.*, 1913, ciii, 695.

query: When does Walden inversion take place? we compared the changes in the optical rotations on passing from the thiols to the sulfonic acids corresponding to a given carbinol.

It is seen from Columns 4 and 5 of Table I that in all cases save two the oxidation of the mercaptans to the sulfo acids leads to a change in the direction of rotation. In all these cases it is assumed that the respective carbinols are configurationally related to the corresponding halides which rotate in the opposite direction, inasmuch as the differences in the polarities of OH → halogen and SH→SO₃H are in the same direction. It was therefore further concluded that in methyl- and ethylphenyl carbinols and in cyclohexylethyl carbinol, thionyl chloride brings about a Walden inversion. It was also concluded that in propyl-, isopropyl-, and butylphenyl carbinols, both reagents bring about an inversion.

The question arises now as to the two exceptional cases; namely, in regard to methylnaphthyl carbinol and cyclohexylphenyl carbinol. In both instances the mercaptans and the corresponding sulfonic acids rotate in the same direction. However, it is noted: First, that on passing from levo-methylnaphthylthiol to the corresponding sulfonic acid, a very high increase in levorotation is observed. Hence it is concluded that levo-methylnaphthyl carbinol and the levo-methylnaphthyl halide are configurationally related. Thus, in this instance, it may be concluded that both reagents bring about a Walden inversion in a manner analogous to their action on propyl-, butyl-, and isobutylphenyl carbinols. Second, cyclohexylphenyl thiol, on passing to the sulfonic acid, shows a change of rotation in the opposite direction, though the rotation remains of the same sign. Hence it is assumed that in this case the carbinol is related to that halide which rotates in the opposite direction, inasmuch as the halide rotating in the same direction as the carbinol has a higher molecular rotation than the carbinol. Therefore, the reagent which on halogenation leaves the direction of rotation unchanged brings about a Walden inversion. Thus, it would seem as if the reaction proceeds in a manner analogous to the case of methyl- and ethylphenyl carbinols; namely, that thionyl chloride brings about a Walden inversion. It is realized, however, that the above conclusions need to be tested by other methods and we hope to

be able to do so in the near future. If the present argument is substantiated, then the following conclusions will be justified. (1) Halogenation of simple aliphatic secondary alcohols as well as of benzyl and phenyl carbinols proceeds without Walden inversion. (2) In methyl-, ethyl-, and cyclohexylphenyl carbinols only thionyl chloride brings about the inversion. (3) In n-propyl-, isopropyl-, and n-butylphenyl carbinols and in methylnaphthyl carbinol, halogenation always proceeds with a Walden inversion.*

<div align="center">EXPERIMENTAL.</div>

Derivatives of n-Propylmethyl Carbinol.

Resolution of n-Propylmethyl Carbinol.—The resolution was carried out according to the method of Pickard and Kenyon[3] with slight modifications.

100 gm. of n-propylmethyl carbinol were dissolved in 150 cc. of dry pyridine. To this solution were added 175 gm. of phthalic anhydride and the mixture was allowed to stand overnight at room temperature. To complete the reaction the mixture was finally heated for $\frac{1}{2}$ hour on the steam bath. To isolate the ester, ice was added and the solution was treated with an excess of hydrochloric acid. The ester was extracted with chloroform, washed with water, and dried over sodium sulfate. The chloro-

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* *Note on reading proof.* In a paper by Levene and Walti an outline was given of the possible mechanism of the Walden inversion. It was there suggested that the occurrence or non-occurrence of Walden inversion during a reaction of substitution is determined by the relative velocities of two reactions: One may be termed external, a reaction between the group to be substituted and the substituting group; the other may be termed internal, a reaction between two groups within the molecule. It was then pointed out that the assumption lends itself to experimental test; that is, the velocity of the external reaction can be measured by the rate of the reaction of substitution of a halogen atom by an alcoholic group, or of the hydroxyl-hydrogen atom of an alcohol by an acyl group. Results of measurements of the velocities of reactions of the latter type have just been published by J. F. Norris and F. Cortese (*J. Am. Chem. Soc.*, 1927, xlix, 2640). On comparing the velocities of acylation of a long series of alcohols, these authors find the lowest velocities for methyl-, ethyl-, and propylphenyl carbinols. It is significant that in this series of alcohols halogenation results in a Walden inversion.

[3] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 45.

form was then removed under reduced pressure and the residue dissolved in a slight excess of sodium carbonate solution. The trace of unchanged carbinol was removed by extraction with ether. The aqueous layer was then acidified and the ester extracted with ether. On removal of the ether the residue solidified. When purified by recrystallization from ether it melted at 62–63°C. The yield was 252 gm.

The phthalate was converted into the strychnine salt in acetone solution in the usual way. It was repeatedly extracted with hot acetone until constant activity of the phthalate was reached.

$$[\alpha]_D^{20} = \frac{+ 7.14° \times 100}{1 \times 17.56} = + 40.7°. \quad [M]_D^{20} = + 95.6°.$$

The above ester was then steam-distilled with 3 mols of sodium hydroxide. The distillate was extracted with ether and dried over sodium sulfate. The ether was then removed and the residue distilled under atmospheric pressure. The fraction boiling between 117–118°C. showed a rotation of

$$[\alpha]_D^{20} = \frac{+ 1.37° \times 100}{1 \times 7.39} = + 18.5° \text{ (in ether)}. \quad [M]_D^{20} = + 16.3°.$$

Dextro-n-Propylmethylbromomethane.—25 gm. of levo-*n*-propyl-methyl carbinol $[\alpha]_D^{20} = \dfrac{-1.15° \times 100}{1 \times 12.70} = - 9.06°, [M]_D^{20} = -8.0°$ (in ether), were refluxed for $\frac{1}{2}$ hour with 100 cc. of constant boiling hydrobromic acid. The mixture was then steam-distilled and the distillate extracted with ether. The extract was washed with dilute sodium carbonate solution, then with water, and finally dried over sodium sulfate. After the ether had been removed, the residue was distilled under atmospheric pressure. The fraction boiling between 115–118°C. showed a rotation of

$$[\alpha]_D^{20} = \frac{+ 0.98° \times 100}{1 \times 10.72} = + 9.14° \text{ (in ether)}. \quad [M]_D^{20} = + 13.8°.$$

0.1000 gm. substance: 0.1244 gm. AgBr.
$C_5H_{11}Br$. Calculated. Br 52.95. Found. Br 52.94.

Action of Thionyl Chloride on n-Propylmethyl Carbinol.—10 gm. of levo-*n*-propylmethyl carbinol were thoroughly cooled

in a carbon dioxide-ether bath and were then poured into 30 gm. of thionyl chloride which had been cooled in a similar way. The mixture was allowed to stand for $\frac{1}{2}$ hour in the carbon dioxide-ether bath. The excess of thionyl chloride was distilled off under reduced pressure. The residue was then taken up with ice and water and extracted with ether. The extract was washed with dilute sodium hydroxide solution, then with water, and was finally dried over sodium sulfate. The ether was then removed and the residue distilled under reduced pressure. It distilled at 113–119°. From the result of the analysis it is obvious that diamyl sulfite was obtained instead of the expected amyl chloride.

0.1216 gm. substance: 0.1336 gm. $BaSO_4$.

$C_{10}H_{22}O_3S$. Calculated. S 14.41. Found. S 15.09.

$$[\alpha]_D^{20} = \frac{+ 2.22° \times 100}{2 \times 19.88} = + 5.58°. \quad [M]_D^{20} = + 5.9°.$$

Levo-n-Propylmethylmercaptomethane.—20 gm. of dextro-methyl-propylchloromethane

$$[\alpha]_D^{20} = \frac{+ 0.97° \times 100}{1 \times 14.25} = + 6.8° \text{ (in ether), } [M]_D^{20} = + 7.2°$$

were refluxed for 3 hours under a return condenser with 3 mols of alcoholic potassium hydrogen sulfide. Water was then added and the mercaptan extracted with ether. It was washed with water, dried over sodium sulfate, and finally distilled under atmospheric pressure. The fraction boiling at 112°C. showed a rotation of

$$[\alpha]_D^{20} = \frac{- 0.77° \times 100}{1 \times 16.51} = - 4.66° \text{ (in ether). } \quad [M]_D^{20} = - 4.8°.$$

0.1130 gm. substance: 0.2546 gm. $BaSO_4$.

$C_5H_{12}S$. Calculated. S 30.77. Found. S 30.95.

Levo-Pentane-2-Sulfonic Acid.—4 gm. of dextro-*n*-propylmethyl-mercaptomethane

$$[\alpha]_D^{20} = \frac{+ 0.96° \times 100}{1 \times 12.12} = + 7.92° \text{ (in ether), } [M]_D^{20} = + 8.2°$$

were dissolved in 50 cc. of acetone and 2 cc. of water. This solution was then treated with a solution of 14.42 gm. of barium

permanganate in acetone. On addition of the first part of the permanganate the mixture was cooled. When the permanganate was no longer decolorized, the solution was heated on the steam bath until it had all been consumed. The manganese dioxide was then filtered off and washed alternately with water and acetone. The combined filtrates were evaporated to dryness under reduced pressure. The residue was extracted several times with ether to remove impurities and finally recrystallized several times from water.

The salt showed a rotation of

$$[\alpha]_D^{20} = \frac{-0.54° \times 100}{2 \times 10.75} = -2.51°. \quad [M]_D^{20} = -5.51°.$$

For the acid

$$\frac{-0.44° \times 100}{2 \times 6.77} = -3.25°. \quad [M]_D^{20} = -4.9°.$$

0.0962 gm. substance: 0.0508 gm. $BaSO_4$ (for Ba).
0.1282 "   "   : 0.1346 "   "   (" S).
$C_{10}H_{22}S_2O_6$.   Calculated.   Ba 31.27, S 14.60.
                    Found.   " 31.07, " 14.42.

Derivatives of Cyclohexylphenyl Carbinol.

Resolution of Cyclohexylphenyl Carbinol.—100 gm. of cyclohexylphenyl carbinol were dissolved in 150 cc. of dry pyridine. To this were added 77.90 gm. of phthalic anhydride. The mixture was then heated for 2 hours on the steam bath. To isolate the ester the mixture was cooled and treated with an excess of concentrated hydrochloric acid. The ester was extracted with chloroform, washed thoroughly with water, and dried over sodium sulfate. On removal of the chloroform the ester remained behind in a snow-white crystalline form. When recrystallized from ether the racemic form melted at 163°C. The total yield of the ester was 185 gm.

The cinchonidine salt of the ester was prepared by treating a warm solution of 178 gm. of the phthalate in 1000 cc. of acetone with 154 gm. of dry cinchonidine. On cooling, the salt precipitated.

The salt was then extracted twice with warm acetone. On decomposition of the salt an ester was obtained with a rotation of

$$[\alpha]_D^{20} = \frac{+3.10° \times 100}{1 + 7.38} = +42.0° \text{ (in CHCl}_3\text{)}. \quad [M]_D^{20} = +142.0°.$$

This phthalate was recrystallized from ether. It then showed a rotation of

$$[\alpha]_D^{20} = \frac{+2.10° \times 100}{1 \times 4.00} = +52.5° \text{ (in CHCl}_3\text{)}. \quad [M]_D^{20} = +177.4°.$$

Further recrystallization did not increase the optical activity.

The ester was refluxed with 2.5 mols of potassium hydroxide for ½ hour. It was then extracted with ether, dried over sodium sulfate, and finally recrystallized from petrolic ether. It showed an optical rotation of

$$[\alpha]_D^{20} = \frac{+5.88° \times 100}{1 \times 15.63} = +37.6° \text{ (in ether)}. \quad [M]_D^{20} = +71.2°.$$

Action of Thionyl Chloride on Levo-Cyclohexylphenyl Carbinol.— 10 gm. of cyclohexylphenyl carbinol

$$[\alpha]_D^{20} = \frac{-1.42° \times 100}{1 \times 6.64} = -21.4° \text{ (in ether)}, \quad [M]_D^{20} = -40.7°$$

were added slowly with cooling to 40 cc. of thionyl chloride. The mixture was then heated for 15 minutes on the steam bath, the excess thionyl chloride was removed under reduced pressure, and the residue was taken up with ice and water. The chloro derivative was extracted with ether, washed first with dilute sodium carbonate, then with water, and finally dried over sodium sulfate. When the ether was removed, all the residue distilled under a pressure of 0.7 mm. at 105–107°C. and showed a rotation of

$$[\alpha]_D^{20} = \frac{-2.91° \times 100}{1 \times 9.89} = -29.4° \text{ (in ether)}. \quad [M]_D^{20} = -61.3°.$$

0.1116 gm. substance: 0.0774 gm. AgCl.
$C_{13}H_{16}Cl$. Calculated. Cl 17.02. Found. Cl 17.15.

Action of Phosphorus Pentachloride on Levo-Cyclohexylphenyl Carbinol.—5 gm. of levo-cyclohexylphenyl carbinol

$$[\alpha]_D^{20} = \frac{-1.42° \times 100}{1 \times 6.64} = -21.4° \text{ (in ether)}, \quad [M]_D^{20} = -40.7°$$

were dissolved in 10 cc. of dry chloroform.   This solution was then poured with cooling into a suspension of 5.2 gm. of phosphorus pentachloride in 50 cc. of dry chloroform. $\frac{1}{2}$ hour later the chloroform and oxychloride were removed under reduced pressure and the residue was taken up in ice-cold water.   The chloride was purified as described in the previous experiment.   Under a pressure of 1.3 mm. it distilled at 112–113°C. and rotated polarized light to the left.

$$[\alpha]_D^{20} = \frac{-\ 0.51° \times 100}{1 \times 14.88} = -\ 3.43° \text{ (in ether)}. \quad [M]_D^{20} = -\ 7.15°.$$

0.1292 gm. substance: 0.0846 gm. AgCl.
$C_{13}H_{16}Cl$. Calculated. Cl 17.02. Found. Cl 16.20.

Cyclohexylphenylmercaptomethane.—   Levo-cyclohexylphenyl-chloromethane

$$[\alpha]_D^{20} = \frac{-\ 4.17° \times 100}{1 \times 14.28} = -\ 29.2° \text{ (in ether)}, \ [M]_D^{20} = -\ 60.9°$$

was refluxed with 3 mols of alcoholic potassium hydrogen sulfide for 8 hours.   Water was then added and the mercaptan was extracted with ether.   It could not be distilled without decomposition even under high vacuum.   It was, therefore, dissolved in alcohol and fractionally precipitated with water.   A fraction was obtained which gave a rotation of

$$[\alpha]_D^{20} = \frac{+\ 2.84° \times 100}{1 \times 10.24} = +\ 27.7° \text{ (in ether)}. \quad [M]_D^{20} = +\ 57.0°$$

*Cyclohexylphenylmethane Sulfonic Acid.—*15 gm. of dextro-cyclohexylphenylmercaptomethane

$$[\alpha]_D^{20} = \frac{+\ 5.07° \times 100}{1 \times 12.63} = +\ 40.1° \text{ (in ether)}, \ [M]_D^{20} = +\ 82.6°.$$

were oxidized by potassium permanganate in acetone solution containing a little water.   The manganese dioxide was filtered off and washed with acetone and then with water.   The combined filtrates were evaporated to dryness and the residue was washed with ether to remove the ether-soluble impurities.   The residue was then recrystallized twice from water.   The optical activity

of the free sulfonic acid was determined in the presence of an excess of hydrochloric acid.

For free acid,

$$[\alpha]_D^{20} = \frac{+\ 1.34° \times 100}{2 \times 13.97} = +\ 4.79°. \quad [M]_D^{20} = +\ 12.2°.$$

For salt,

$$[\alpha]_D^{20} = \frac{+\ 0.57° \times 100}{2 \times 6.95} = +\ 4.10°. \quad [M]_D^{20} = +\ 12.0°.$$

0.0859 gm. substance: 0.0260 gm. K_2SO_4.
0.0984 "   "   : 0.0760 " $BaSO_4$.

$$C_{13}H_{17}O_3SK. \quad \text{Calculated.} \quad K\ 13.38,\ S\ 10.95.$$
$$\text{Found.} \qquad "\ 13.58,\ "\ 11.19.$$

Derivatives of Methyl-α-Naphthyl Carbinol.

Resolution of Methyl-α-Naphthyl Carbinol.—The resolution was carried out according to the method of Pickard and Kenyon[4] with slight modification.

100 gm. of methyl-α-naphthyl carbinol were dissolved in 200 cc. of dry pyridine. To this solution were added 90 gm. of finely pulverized phthalic anhydride. The mixture was allowed to stand overnight at room temperature. The next day the mixture was heated for 15 minutes on the steam bath to complete the reaction. It was then cooled and acidified with 200 cc. of concentrated hydrochloric acid. The half ester which separated was extracted with chloroform. The extract was washed with water and dried over sodium sulfate. The chloroform was then removed under reduced pressure. To purify the residue, it was treated with an excess of sodium carbonate in water solution. The solution was then extracted with ether to remove the unchanged carbinol. The aqueous layer was then acidified and the ester extracted with ether. The residue which remained behind on removal of the ether was sufficiently pure for resolution. To effect this, the ester was converted into the strychnine salt in methylethyl ketone solution. The salt which crystallized almost immediately was filtered off while the solution was still hot. This salt was extracted twice with hot methylethyl ketone and it was then decomposed in the

[4] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1914, cv, 1126.

usual way with hydrochloric acid.   The half ester thus obtained showed a rotation of

$$[\alpha]_D^{20} = \frac{+\ 2.88° \times 100}{1 \times 7.64} = +\ 37.7° \text{ (in chloroform)}. \quad [M]_D^{20} = +\ 120.6°.$$

To obtain the carbinol from the above phthalate, the latter was heated to boiling for about 2 minutes with 3 mols of dilute sodium hydroxide.   The carbinol was then extracted with ether, washed with water, and dried over sodium sulfate.   On removal of the ether the residue showed a rotation of

$$[\alpha]_D^{20} = \frac{-\ 3.28° \times 100}{1 \times 4.70} = -\ 69.8° \text{ (in alcohol)}. \quad [M]_D^{20} = -\ 120.0°.$$

Action of Thionyl Chloride on Methyl-α-Naphthyl Carbinol.— 35 gm. of dextro-methyl-α-naphthyl carbinol, which showed a rotation of $[\alpha]_D^{20} = +11.06°$, were dissolved in 70 cc. of dry ether. This solution was poured slowly, with thorough cooling, into a mixture of 140 cc. of thionyl chloride and 150 cc. of dry ether. The mixture was allowed to stand at room temperature for ½ hour, whereupon the ether and the excess of thionyl chloride were removed under reduced pressure at 50°C.   The residue was then treated with ice and water and extracted with ether.   The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate.   The ether was then removed and the residue dissolved in petrolic ether.   On cooling in a carbon dioxide-ether bath the chloro derivative separated as an oil. This treatment was repeated several times.   The chloride finally precipitated in a snow-white crystalline form which melted at room temperature.   When pure the substance showed a rotation of

$$[\alpha]_D^{20} = \frac{-\ 3.38° \times 100}{1 \times 9.28} = -\ 36.4° \text{ (in ether)}. \quad [M]_D^{20} = -\ 69.4°.$$

0.1436 gm. substance: 0.1076 gm. AgCl.
$C_{12}H_{11}Cl$.   Calculated.   Cl 18.62.   Found.   Cl 18.53.

Action of Phosphorus Pentachloride on Levo-Methyl-α-Naphthyl Carbinol.—3 gm. of levo-methyl-α-naphthyl carbinol with an optical rotation of

$$[\alpha]_D^{20} = \frac{-\ 2.42° \times 100}{1 \times 6.88} = -\ 35.2° \text{ (in alcohol)}, \quad [M]_D^{20} = -\ 60.5°$$

were dissolved in 10 cc. of dry chloroform. The solution was thoroughly cooled and then poured slowly with cooling into a suspension of 3.6 gm. of phosphorus pentachloride in 20 cc. of dry chloroform. The mixture was allowed to stand for half an hour at room temperature, whereupon the chloroform and phosphorus oxychloride were removed at 40°C. under reduced pressure. The residue was poured into a little crushed ice and extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The chloro derivative showed a rotation of

$$[\alpha]_D^{20} = \frac{+\ 0.96° \times 100}{1 \times 17.7} = +\ 5.42° \text{ (in ether).} \quad [M]_D^{20} = +\ 10.3°.$$

The substance was analyzed without further purification.

0.1460 gm. substance: 0.0964 gm. AgCl.
          $C_{12}H_{11}Cl$. Calculated. Cl 18.62.   Found. Cl 16.33.

Dextro-Methyl-α-Naphthylmercaptomethane.—20 gm. of levo-methyl-α-naphthylchloromethane

$$[\alpha]_D^{20} = \frac{-\ 0.60° \times 100}{1 \times 4.96} = -\ 12.1°, [M]_D^{20} = -\ 23.1°$$

were heated with 3 mols of alcoholic potassium hydrogen sulfide for 3 hours under a return condenser. The solution was then poured into crushed ice and water, and the mercaptan was extracted with ether.   The extract was washed with water and dried over sodium sulfate.   The ether was then removed and the residue dissolved in petrolic ether. The solution was cooled in a carbon dioxide-ether bath.   When a little of the oil separated, the ether layer was poured off.   This ethereal solution was concentrated and cooled and the ether layer was again poured off. Finally, the ether layer was concentrated still more.   On thorough cooling, a crystalline precipitate was obtained, which was filtered off.   The filtrate was evaporated to dryness under reduced pressure.   The residue showed a rotation of

$$[\alpha]_D^{20} = \frac{+\ 0.96° \times 100}{1 \times 10.45} = +\ 9.2°. \quad [M]_D^{20} = +\ 17.3°.$$

0.1362 gm. substance: 0.1580 gm. BaSO₄.
          $C_{12}H_{12}S$. Calculated. S 17.02.   Found. S 15.94.

Methyl-α-Naphthylmethyl   Sulfonic   Acid.—10   gm.   of   levo-methyl-α-naphthylmercaptomethane

$$[\alpha]_D^{20} = \frac{- 3.06° \times 100}{1 \times 12.33} = - 24.8° \text{ (in ether)}, \ [M]_D^{20} = - 46.6°$$

were dissolved in 400 cc. of acetone and 20 cc. of water.   10 gm. of potassium permanganate in acetone solution were then added slowly.   Finally the solution was heated on the steam bath to complete the reaction.   The manganese dioxide was filtered off, the precipitate washed with hot water, and the combined filtrates evaporated to dryness under reduced pressure.   The residue was washed with ether and then recrystallized several times from water. 0.235 gm. of the salt was weighed in a volumetric flask, treated with an excess of hydrochloric acid, and diluted to 10 cc.   The amount of salt taken corresponds to 0.202 gm. of free acid.

$$[\alpha]_D^{20} = \frac{- 2.24° \times 100}{1 \times 3.35} = - 66.9°.   \ [M]_D^{20} = - 157.8°.$$

To determine the rotation of the salt, 0.3616 gm. of the salt was dissolved in water and diluted to 10 cc.

$$[\alpha]_D^{20} = \frac{- 1.92° \times 100}{1 \times 3.58} = - 53.6°.   \ [M]_D^{20} = - 146.9°.$$

0.0857 gm. substance: 0.0254 gm. K_2SO_4 (for K).
0.1544   "   "   : 0.1394 " $BaSO_4$ (" S).
$C_{12}H_{11}SO_3K$.   Calculated.   K 14.28, S 11.68.
                    Found.   " 13.30, " 12.40.

Derivatives of Cyclohexylmethyl Carbinol.

Resolution of Cyclohexylmethyl Carbinol.—The resolution was carried out according to the method of Domleo and Kenyon[5] with slight modifications.

100 gm. of cyclohexylmethyl carbinol dissolved in 150 cc. of dry pyridine were heated for 2 hours on the steam bath with 121.3 gm. of phthalic anhydride.   An excess of hydrochloric acid was then added and the ester was extracted with ether.   It was washed free of hydrochloric acid and dried over sodium sulfate.   The

[5] Domleo, A., and Kenyon, J., *J. Chem. Soc.*, 1926, 1841.

solvent was removed and the residue dissolved in a slight excess of sodium carbonate. The unchanged carbinol was removed by extracting with ether. The mother liquor was acidified and the ester extracted with chloroform. When the chloroform was removed the residue crystallized in the form of thick prisms which were easily recrystallized from ether. When pure it melted at 138°C. The yield was 182 gm. The phthalate was converted into the brucine salt in acetone solution, from which it crystallized immediately. The salt was then repeatedly extracted with hot acetone until the ester obtained from the salt no longer showed an increase of rotation.

$$[\alpha]_D^{20} = \frac{+\ 4.38° \times 100}{1 \times 9.68} = +\ 45.3° \text{ (in ether)}. \quad [M]_D^{20} = +\ 125.0°.$$

The ester was then steam-distilled with 2.5 mols of potassium hydroxide. A carbinol was obtained with a rotation of

$$[\alpha]_D^{20} = \frac{+\ 0.77° \times 100}{1 \times 9.14} = +\ 8.43° \text{ (in ether)}. \quad [M]_D^{20} = +\ 10.8°.$$

Action of Phosphorus Pentachloride on Dextro-Cyclohexylmethyl Carbinol.—10 gm. of cyclohexylmethyl carbinol

$$[\alpha]_D^{20} = \frac{+\ 1.37° \times 100}{1 \times 18.52} = +\ 7.40° \text{ (in ether)}, \ [M]_D^{20} = +\ 9.5°$$

were dissolved in 20 cc. of dry chloroform. This solution was then added with cooling to a suspension of 16.25 gm. of phosphorus pentachloride in 100 cc. of dry chloroform. The mixture was then allowed to stand at room temperature for 1 hour, whereupon the chloroform was removed under reduced pressure. The residue was then taken up with ice water, extracted with ether, and the extract washed with dilute sodium hydroxide solution and then with water. When dry, the ether was removed and the residue distilled under reduced pressure (about 16 mm.). Under this pressure the substance distilled at 70–72°C. and showed a rotation of

$$[\alpha]_D^{20} = \frac{-\ 1.52° \times 100}{1 \times 30.27} = -\ 5.02° \text{ (in ether)}, \ [M]_D^{20} = -\ 7.4°.$$

0.1000 gm. substance: 0.0983 gm. AgCl.

$C_8H_{15}Cl$. Calculated. Cl 24.23. Found. Cl 24.32.

Cyclohexylmethylmercaptomethane.—24 gm. of cyclohexylme-
thylchloromethane

$$[\alpha]_D^{20} = \frac{+\ 0.98° \times 100}{1 \times 25.6} = +\ 3.83°, \ [M]_D^{20} = +\ 5.6°$$

were refluxed for 16 hours with 3 mols of alcoholic potassium hy-
drogen sulfide. Water was then added and the mercaptan ex-
tracted with ether, washed with water, and dried over sodium sul-
fate. The ether was then removed and the residue distilled under
reduced pressure. The fraction boiling at 70–80°C. showed a ro-
tation of

$$[\alpha]_D^{20} = \frac{-\ 0.73° \times 100}{1 \times 20.24} = -\ 3.60° \ (in \ ether), \ [M]_D^{20} = -\ 5.2°.$$

Cyclohexylmethylmethane Sulfonic Acid.—1.5 gm. of levo-cyclo-
hexylmethylmercaptomethane

$$[\alpha]_D^{20} = \frac{-\ 0.73° \times 100}{1 \times 20.24} = -\ 3.60°, \ [M]_D^{20} = -\ 5.2°$$

were oxidized with potassium permanganate in acetone solution
containing a little water. To complete the reaction the mixture
was finally heated for a few minutes on the steam bath. The
manganese dioxide was filtered off and washed with water. The
combined filtrates were evaporated to dryness. The crystalline
residue was washed with ether and then recrystallized from alco-
hol. The rotation of the free sulfonic acid was determined in the
presence of excess of hydrochloric acid.

For salt,

$$[\alpha]_D^{20} = \frac{+\ 0.81° \times 100}{2 \times 5.50} = +\ 7.4°. \quad [M]_D^{20} = +\ 16.9°.$$

For acid,

$$\frac{0.70° \times 100}{2 \times 3.85} = +\ 9.1°. \quad [M]_D^{20} = +\ 17.5°.$$

0.0886 gm. substance: 0.0332 gm. K₂SO₄.
0.0980 "   "   : 0.1072 " BaSO₄.
            C₈H₁₅SO₃K. Calculated. K 16.98, S 13.90.
                        Found.   " 16.81, " 15.03.

Derivatives of Cyclohexylethyl Carbinol.

Resolution of Cyclohexylethyl Carbinol.—50 gm. of racemic cyclo-hexylethyl carbinol dissolved in 75 cc. of pyridine were treated with 52 gm. of phthalic anhydride and heated for 2 hours on the steam bath. The ester was isolated and purified in exactly the same way as in the case of the corresponding methylcyclohexyl derivative. The yield was 71 gm., m.p. = 87–89°C.

66 gm. of the phthalate were dissolved in chloroform and treated with 76 gm. of strychnine. On heating, all the alkaloid dissolved. The chloroform was then distilled off under reduced pressure and the residue taken up with hot acetone. The residue crystallized immediately on addition of acetone. The salt was then repeatedly extracted with hot acetone until the phthalate obtained showed no increase in rotation. The highest rotation obtained was

$$[\alpha]_D^{20} = \frac{+\ 1.46° \times 100}{1 \times 13.83} = +\ 10.5° \text{ (in ether).} \quad [M]_D^{20} = +\ 30.2°.$$

The above phthalate was steam-distilled with 3 mols of potassium hydroxide. A carbinol was obtained which showed a rotation of

$$[\alpha]_D^{20} = \frac{-\ 1.18° \times 100}{1 \times 12.28} = -\ 9.60°. \quad [M]_D^{20} = -\ 13.6°.$$

Action of Phosphorus Pentachloride on Dextro-Cyclohexylethyl Carbinol.—5 gm. of cyclohexylethyl carbinol

$$[\alpha]_D^{20} = \frac{+\ 0.96° \times 100}{1 \times 10.7} = +\ 8.95° \text{ (in ether), } [M]_D^{20} = +\ 12.70°$$

dissolved in 10 cc. of dry chloroform were poured with cooling into a suspension of 7.32 gm. of phosphorus pentachloride in 50 cc. of dry chloroform. When all the pentachloride had been added the mixture was allowed to stand at room temperature for 1 hour to complete the reaction. The chloroform and oxychloride were then distilled off under reduced pressure, the residue taken up with ice water and extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was then removed and the residue

distilled under a pressure of about 16 mm.   The fraction boiling between 88–93°C. showed a rotation of

$$[\alpha]_D^{20} = \frac{- \, 0.16° \times 100}{1 \times 13.3} = - \, 1.20° \text{ (in ether)}. \quad [M]_D^{20} = - \, 1.92°.$$

0.1432 gm. substance: 0.1298 gm. AgCl.
      $C_9H_{17}Cl$.   Calculated.   Cl 22.10.   Found.   Cl 22.42.

Action of Thionyl Chloride on Dextro-Cyclohexylethyl Carbinol.— 15 gm. of dextro-cyclohexylethyl carbinol were dissolved in 30 cc. of dry ether and thoroughly cooled in a carbon dioxide-ether bath. This solution was then poured into 45 cc. of thionyl chloride which also had previously been cooled in a carbon dioxide-ether bath. When all the carbinol had been added, the mixture was allowed to stand in the carbon dioxide-ether bath for $\frac{1}{2}$ hour.   It was then poured into ice water and extracted with ether.   The extract was washed first with dilute sodium hydroxide, then with water, and it was finally dried over sodium sulfate.   The solvent was then removed and the residue was fractionated under a pressure of about 16 mm.   The fraction boiling at 90–91°C. showed a rotation of

$$[\alpha]_D^{20} = \frac{- \, 1.02° \times 100}{2 \times 17.2} = - \, 2.97°. \quad [M]_D^{20} = - \, 4.8°.$$

The chloro derivative analyzed as follows:

0.1234 gm. substance: 0.0830 gm. AgCl.
      $C_9H_{17}Cl$.   Calculated.   Cl 22.10.   Found.   Cl 16.64.

Cyclohexylethylmercaptomethane.—12 gm. of levo-cyclohexyl-ethylchloromethane

$$[\alpha]_D^{20} = \frac{- \, 0.34° \times 100}{2 \times 18.42} = - \, 0.92°, \; [M]_D^{20} = - \, 1.48°$$

were heated with 3 mols of alcoholic potassium hydrogen sulfide in a pressure bottle for 14 hours at 100°C.   The solution was then poured into cold water and extracted with ether.   The extract was washed and then dried over sodium sulfate.   The solvent was

then removed and the residue distilled under a pressure of about 16 mm.   The fraction boiling at 95--100°C. was dextrorotatory.

$$[\alpha]_D^{20} = \frac{+\ 0.16° \times 100}{2 \times 12.49} = +\ 0.64°. \quad [M]_D^{20} = +\ 1.10°.$$

It analyzed as follows:

0.0905 gm. substance: 0.1111 gm. BaSO₄.
$C_9H_{18}S$. Calculated. S 20.23. Found. S 16.86.

Ethylcyclohexylmethane Sulfonic Acid.—2 gm. of dextro-ethyl-cyclohexylmercaptomethane

$$[\alpha]_D^{20} = \frac{+\ 0.21° \times 100}{2 \times 22.5} = +\ 0.93°, [M]_D^{20} = +\ 1.47°$$

were dissolved in 30 cc. of acetone and 5 cc. of water.   This was then treated with 4.74 gm. of barium permanganate in acetone solution.   The reaction was finally brought to completion by heating on the steam bath for ½ hour.   The manganese dioxide was filtered off and washed with water.   The filtrate was evaporated to dryness under reduced pressure.   The residue was first washed with ether and then dissolved in a very small amount of water. Alcohol was then added, whereupon the salt precipitated.   It was then redissolved in water and reprecipitated with alcohol.   It was dextrorotatory.

For salt,

$$[\alpha]_D^{20} = \frac{+\ 0.21° \times 100}{2 \times 5.86} = +\ 1.79°. \quad [M]_D^{20} = +\ 4.7°.$$

For acid,

$$[\alpha]_D^{20} = \frac{+\ 0.22° \times 100}{2 \times 3.70} = +\ 2.97°. \quad [M]_D^{20} = +\ 7.7°.$$

ON SOME NEW LIPOIDS.

BY P. A. LEVENE AND K. LANDSTEINER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

In course of an investigation undertaken by Landsteiner and Levene[1] aiming at the isolation of the heterogenetic haptene from that fraction of the lipoids of the horse kidney which often had been termed "white matter," a material was obtained with peculiar properties hitherto not observed on other known lipoids.

The peculiarities of the material were the following. (1) As originally obtained, the substance was water-soluble, giving a viscous solution in concentration of about 2 per cent. (2) On heating on the water bath with orcinol, hydrochloric acid, and a drop of copper acetate, in place of the green coloration obtained on heating ordinary carbohydrates, a characteristic purplish red coloration was obtained. The usual green coloration was obtained if, prior to treatment with orcinol, the substance was hydrolyzed with mineral acids. (3) The elementary composition of the substance differed markedly from cerebrosides and from sphingomyelin. The elementary composition, however, varied for different samples. Table I gives the analytical data of substances from different sources. It is seen from Table I that the material was obtained not only from horse kidney, but also from beef kidney and from beef brain.

The above three peculiarities distinguish the new material from the known lipoids. In common with the latter, it gives on hydrolysis fatty acids, sphingosine, and in common with some, a sugar, most probably galactose.

The material with these properties was described in several publications by Landsteiner and Levene. Seemingly, these were not

[1] Landsteiner, K., and Levene, P. A., *J. Immunol.*, 1925, x, 731; *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 343; 1927, xxiv, 693.

noticed by workers in the field of lipoids inasmuch as very recently Walz[2] in a publication from Thierfelder's laboratory announced the discovery of a new lipoid which was characterized by the peculiar orcinol test, mentioned above. Our own investigation into the nature of the new substance is only in its initial phase and the results are reported now partly because of the publication from Thierfelder's laboratory and partly for the reason that it has already been possible to separate the original material into three fractions, each having an interest of its own.

Fractionation of the Crude Material.—The crude material was fractionated into three parts.

1. Free from Sulfur and Phosphorus.—This substance was characterized by its behavior towards Fehling's solution in the cold; namely, an aqueous solution of the material on addition of

TABLE I.

	C	H	N	P	S	Ash.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Horse Kidney 1......	59.64	10.09	2.33	1.19	0.81	4.22
" " 2......	55.92	8.83	2.75	1.58	1.98	11.55
Beef kidney..........	53.79	8.38		0.47	0.43	6.58
Beef Brain 273⅜⅝.....	57.14	9.10	2.27	1.34	1.35	10.37

Fehling's solution gave a bluish precipitate only slightly soluble in water but soluble in an excess of mineral acid. The behavior is analogous to that of yeast gum. In the present state of purity the substance still contains the components of lipoids. This substance is described in a preliminary way by Landsteiner and Levene in another place.

2. Free from Phosphorus but Containing Sulfur.—One of us (Levene[3]) was only once successful in the preparation of a sulfatide free from phosphorus. Later efforts by the same author to prepare it were not successful, as great difficulties were encountered in removing the last traces of phosphorus-containing substances. The present sulfur-containing and phosphorus-free

[2] Walz, E., *Z. physiol. Chem.*, 1927, clxvi, 210.
[3] Levene, P. A., *J. Biol. Chem.*, 1912–13, xiii, 463.

material was obtained twice by procedures differing somewhat in detail, but similar in principle.

3. Free from Sulfur but Containing Phosphorus.—This substance gives the characteristic orcinol test described above.

<div align="center">TABLE II.</div>

Fraction No.	C	H	N	P	S	Orcinol test.
	per cent	per cent	per cent	per cent	per cent	
1a	55.39	9.22	2.15	0	0	Green.
b	40.91	6.78	1.79	0	0	"
2a	60.28	8.16	1.63	0	2.55	"
b	56.30	9.35	1.59	0	2.87	"
3*	43.69	7.76	0.95	1.89	0	Strong purple.

* This sample contained nearly 30 per cent of mineral material. The analytical values are calculated ash-free.

The elementary composition of various samples belonging to the three fractions was given in Table II.

From the analytical data it is evident that the fractions did not yet represent pure individual substances.

The peculiarities of each fraction and the properties common to all are as follows:

On hydrolysis, they all yield a reducing substance and a water-insoluble residue soluble in organic solvents.

The fractions described as Fractions 1 and 3 are soluble in water on addition of alkali and in this respect they differ from Fraction 2.

Fraction 2 is but slightly soluble in water.

Fractions 1 and 2 differ from Fraction 3 in that they give with orcinol the usual green coloration, whereas Fraction 3 gives an intense purplish red coloration.

Thus far the fractionations were accomplished only on material obtained from horse kidney. However, from beef brain and from

beef kidney materials were obtained which had the same properties and the same elementary composition as the crude material obtained from the horse kidney and therefore it is hoped that similar fractionation will be successfully carried out on the material from beef brain and from other organs.

SUMMARY.

1. From the so called "white matter" from horse kidney, from beef brain, and from beef kidney, a material was obtained with hitherto undescribed properties and with an elementary composition differing from that of the known lipoids.

2. The material obtained from the horse kidney was fractionated into three parts: (*a*) Precipitable from an aqueous solution by Fehling's solution in a manner similar to animal gum. It contains neither sulfur nor phosphorus and is soluble in aqueous alkali. (*b*) Contains sulfur, but does not contain phosphorus; is not soluble in water. (*c*) Does not contain sulfur but contains phosphorus. It is soluble in aqueous alkali, not soluble in water, and gives a very characteristic test with orcinol and copper.

EXPERIMENTAL.

In the earlier phases of the work the material containing the three substances was obtained in one fraction which was subsequently separated into three parts. With the progress of the work it was found advantageous at the very start to separate the so called white matter into two fractions, one containing, among other things, Fraction 1 and the other containing, among other things, Fractions 2 and 3.

Separation of the White Material into Two Fractions.—In a general way the process was based on the solubility of Fraction 1 in pyridine. This solubility is not the property of Fraction 1 in pure condition but of Fraction 1 in the presence of all the other material contained in the white matter.

In detail the procedure is the following. 100 gm. of the white matter are taken up in 500 cc. of boiling pyridine and the mixture is allowed to stand at 0°C. for 48 hours. The residue is then dissolved in 1000 cc. of hot pyridine and allowed to cool at 0°C. for 48 hours. The operation is repeated once, and thus two frac-

tions are obtained, one pyridine-soluble and the other pyridine-insoluble.

Pyridine-Soluble Fraction.—This fraction contains Fraction 1. It is concentrated to dryness, the residue is taken up in 1 part of a solution consisting of equal parts of chloroform and methyl alcohol, and the mixture is brought to a boil. The insoluble part contains Fraction 1. It is purified by precipitation from an aqueous solution by means of Fehling's solution. The details of the procedure are given in an article by Landsteiner and Levene[4] and hence will be omitted here until the more detailed work on the structure of the substance is completed. ·

Pyridine-Insoluble Fraction.—This fraction contains Fractions 2 and 3. Several procedures were employed. Only the one which for the present seems to us the most satisfactory is described in this place.

The dry residue is washed with acetone and ether until the odor of pyridine is entirely removed. The air-dry residue, 75 gm. in weight, is taken up in 150 cc. of chloroform and 75 cc. of methyl alcohol, and the mixture is placed on a boiling water bath where it is allowed to remain until the greater part is dissolved, leaving a comparatively small residue adhering to the wall of the flask. This residue is reextracted on the boiling water bath with 110 cc. of a solution containing 2 parts of chloroform to 1 of methyl alcohol. The final residue is water-soluble, gives the characteristic orcinol test, and analyzes as given in the introductory part for the crude substances from any one of the three organs tested. The yield of this crude material is 10 per cent of the pyridine-insoluble fraction.

Further Fractionation of the Crude Material.—The separation of the phosphorus-free sulfur-containing material is based on the differences in the solubilities in glacial acetic acid and in dilute aqueous alkali. The crude material is dissolved in hot glacial acetic acid in proportion of 1 gm. to 5 cc. The solution is allowed to cool to room temperature and filtered. The yield of the insoluble fraction is about 30 per cent of the starting material. The operation is repeated and the material insoluble in glacial acetic acid is freed from adhering acid by washing with acetone.

[4] Landsteiner, K., and Levene, P. A., *J. Immunol.*, 1927, xiv, 81.

The air-dry residue is then taken up in 5 parts of 5 per cent sodium hydroxide solution and shaken in a shaking machine for 1 hour. The mixture is then centrifugalized and the insoluble residue again dissolved in hot glacial acetic acid and allowed to cool. This residue is washed with acetone to remove the adhering glacial acetic acid and analyzed.

15.6   mg. substance: 0.2556 mg. N (micro method of Van Slyke).
0.1286 gm.   "   : 0.0226 gm. $BaSO_4$.
3.057 mg.   "   gave on Pregl micro combustion 6.758 mg. CO_2 and
        2.23 mg. H_2O.

C 60.28, H 8.16, N 1.63, S 2.55.

All the mother liquor from the fraction insoluble in glacial acetic acid was concentrated to small volume and then poured into acetone. The precipitate obtained in this manner was taken up in dilute aqueous alkali and shaken for 1 hour. The mixture which contained a very slight turbidity was filtered over charcoal. The filtrate was again concentrated to small volume and poured into acetone. A precipitate formed. The latter was taken up in hot glacial acetic acid. The mixture was slightly turbid. It was rapidly centrifugalized in order to avoid cooling. From the supernatant liquid on cooling a precipitate settled out which analyzed as follows:

17.70   mg. substance: 0.2805 mg. N.
0.1000 gm.   "   : 0.0200 gm. $BaSO_4$.
4.807 mg.   "   : 9.924 mg. CO_2 and 4.021 mg. H_2O.

C 56.30, H 9.35, N 1.59, S 2.87.

The filtrate was concentrated to dryness, dissolved in chloroform, and the solution poured into an excess of acetone. A precipitate formed which analyzed as follows:

0.0376 gm. substance: 0.044 gm. $Mg_2P_2O_7$.
14.11   mg.   "   : 0.153 cc. N_2 (micro Van Slyke).
3.422   "   "   : 5.484 mg. CO_2 and 2.435 mg. H_2O.

C 43.69, H 7.76, N 0.95, P 1.89.

This substance gives the characteristic color test with orcinol and copper.

THE OCCURRENCE OF A CELLULASE IN THE SHIP-WORM.

BY LYMAN C. BOYNTON AND ROBERT C. MILLER.

(From the Departments of Chemistry and Zoology, University of Washington, Seattle.)

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The marine wood-boring mollusks of the family Teredinidæ, commonly called Teredos or ship-worms, are well known for their destructive action on wharf piling and other timbers exposed in sea water. Whether the organisms bore in wood for protection only, or whether they also use the wood as food, was a debated question, until it was shown by Dore and Miller (1923) that about 80 per cent of the cellulose, and from 15 to 56 per cent of the hemicelluloses, in Douglas fir piling disappear from the wood during its passage through the digestive tract of *Teredo navalis.* The conclusion was drawn that the substances which disappear are hydrolyzed to simple carbohydrates which the animal can use as food. This conclusion was confirmed by Miller and Boynton (1926), who found in the case of a related organism, *Bankia setacea,* that wood removed from the digestive tract (cecum) contained about four times the amount of reducing sugars present in the original wood.

These findings constitute presumptive evidence of an enzyme in the digestive tract of the ship-worm which is capable of splitting cellulose; but positive proof of the presence of such an enzyme has heretofore been lacking. Harington (1921) has demonstrated that an extract of the liver of *Teredo norvegica* is capable of hydrolyzing starch completely to glucose, and has found some evidence of a partial hydrolysis of sawdust, but no evidence of hydrolysis of pure cellulose (filter paper). He considers his results inconclusive, however, in regard to the sawdust and cellulose, as shortage of material forced him to use small concentrations of enzyme and prevented his running rigorous controls. The present writers

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have followed out the line of investigation suggested by Haring-ton's work, using larger concentrations of enzyme and a different technique.

100 specimens of the Northwest ship-worm (*Eankia setacea*), averaging between 1 and 2 feet in length, were secured from piling at the Puget Sound Biological Station. From 50 of these, after removal of the shell and as much of the posterior adductor muscle as adhered to it, an extract was made of 2 to 3 cm. of the anterior end of the organism, including esophagus, stomach, crystalline style, the so called "livers" or digestive diverticula, and a few mm. of the anterior end of the cecum; the foot, and portions of the mantle, posterior adductor muscle, kidney, etc., were also in-cluded, as their presence was not regarded as particularly objec-tionable, and it was feared that, after the organisms had been re-moved from the wood, changes in the secretory processes might occur in the time required for a careful dissection of this number of specimens. From the remaining 50 specimens the crystalline styles were removed and extracted separately, while at the same time an extract was made of the anterior ends of these individuals after the styles had been taken out.

In each case the extract was prepared by grinding the material in a mortar with clean sand, shaking it up with a solution of 30 per cent alcohol buffered at about pH 6, and allowing to stand 3 days, with frequent shaking. This concentration of alcohol was selected as being sufficient to prevent ordinary bacterial action, without at the same time precipitating the enzymes that might be present. The solution was buffered rather arbitrarily at pH 6, Yonge (1925) having shown that the digestive tract of lamellibranchs is more or less acid in reaction.

After 3 days the preparations were centrifuged, and the super-natant liquid, constituting a stable colloid, was poured off and made up in each case to 150 cc. with additional alcoholic buffer solution. Finally, 50 cc. of the style extract and 50 cc. of the extract from which the styles had been excluded were mixed, giving thus a total of four preparations (A, B, C, D), which were divided among test-tubes and subjected to the experiments outlined in Table I.

The sawdust used was Douglas fir, dried and ground to pass a screen having 100 meshes to the linear inch. The filter paper was

a high grade, acid-washed paper, likewise ground to pass the 100 mesh screen.   All tubes were kept at room temperature, with frequent shaking.   At the end of the 1st week and at the end of the

TABLE I.

Results of Digestion of Sawdust and Cellulose by Enzymes of Bankia.

Reducing sugars expressed as mg. of copper in cuprous oxide formed by reduction of Fehling's solution.

	Sugar present in 5 cc. sample after 3 wks.	Amount of sugar formed by digestion (5 cc. sample).
A. Extract of entire anterior end.		
20 cc. extract without substrate (control for autolysis).....................................	224.6	
20 cc. extract + 200 mg. sawdust..............	234.3	9.7
Same, boiled to destroy enzymes..............	147.2	
20 cc. extract + 200 mg. filter paper...........	229.4	4.8
Same, boiled to destroy enzymes..............	99.2	
B. Extract of crystalline styles.		
20 cc. extract without substrate (control for autolysis)....................................	0.6	
20 cc. extract + 200 mg. sawdust................	1.2	None.
Same, boiled to destroy enzymes................	1.1	
20 cc. extract+200 mg. filter paper (pure cellulose).	0.6	None.
C. Extract of anterior end without styles.		
20 cc. extract without substrate (control for autolysis)....................................	202.8	
20 cc. extract + 200 mg. sawdust................	227.5	24.7
Same, boiled to destroy enzymes	144.3	
20 cc. extract + 200 mg. filter paper.............	210.4	7.6
Same, boiled to destroy enzymes..............	128.7	
D. Extracts B and C mixed in equal proportions.		
20 cc. extract without substrate (control for autolysis)....................................	104.2	
20 cc. extract + 200 mg. sawdust..............	115.8	11 6
20 " " + 200 " filter paper............	108.4	4.2
Same, boiled to destroy enzymes................	67 3	

3rd week the amount of reducing sugars in a 5 cc. sample from each tube was determined by the Munson-Walker general method, with determination of reduced copper by the volumetric thiosulfate method, as described by the Association of Official Agri-

cultural Chemists (1925). In determining the small amounts of sugar present in the style extract (B), a micro burette was used in the final titration.

Definite evidence of digestion of both sawdust and filter paper by Extracts A, C, and D, was obtained at the end of the 1st week; but, as further digestion occurred thereafter, the results at the end of 3 weeks only are given in Table I. The amount of sugar formed by digestion of the substrate in the unboiled tubes is obtained by subtracting from the amount present in the sample the amount found in the control for autolysis. The boiled controls show merely that digestion was stopped by destruction of the enzyme; the sugar in these tubes is to be accounted for chiefly by autolysis during the preparation of the extract.

The extract of crystalline styles (B) gave no evidence of any effect on either sawdust or cellulose, the slightly larger amounts of sugar in the tubes containing sawdust being no more than would be present in the sawdust itself when added. The results in the case of the extract of the anterior end with the styles removed (C), however, indicate a considerable digestion of the sawdust, and a definite, although less marked, digestion of pure cellulose in the form of filter paper.

Evidence of digestion of sawdust and filter paper was also obtained with the extract of the entire anterior end (A) and with the mixed extract (D), which should have had the same properties as Extract A but was only about one-half as concentrated. The amount of sugar formed by digestion of the substrate was about the same in these two cases, although much less sugar was formed by autolysis in the less concentrated extract. In both Extracts A and D the amounts of sugar formed were very much less than in Extract C. The reason for this has not been ascertained; but it seems definitely to eliminate the possibility that the style contains a coenzyme used in the digestion of wood, as the process apparently goes on more successfully in the absence of the style extract than in its presence.

In the case of Extract C we have a fair degree of activity of the enzyme, under conditions that are presumably not optimum. It should be noted that the figures given are all on the basis of a 5 cc. sample. If we assume the sugar to be glucose, an approximate calculation indicates that about 23 per cent by weight of the saw-

dust, and about 7 per cent by weight of the filter paper, have been converted into sugar by digestion. The percentage of sugar formed from the sawdust is as great as that commonly obtained in the saccharification of wood by dilute acids.

The possibility should be considered that the results are due to the action of symbiotic bacteria in the digestive tract of the ship-worm rather than to enzymes produced by the organism itself. Schorger (1926, p. 476) states regarding the cellulose-splitting bacteria, "To secure fermentation, it is necessary that the bacteria be in direct contact with the cellulose." It is regarded as extremely improbable that a bacterium symbiotic in the digestive tract of *Bankia* would continue to live and operate in a solution of 30 per cent alcohol. Furthermore, there was no evidence of bacterial action in any of our tubes, except the development of yeasts in two of the *boiled* controls.

Unfortunately, none of the above preparations were subjected to adequate bacteriological examination. Subsequently, however, an extract of the anterior ends of fifteen specimens of *Bankia*, prepared in the same way, was very kindly examined by Dr. Rachel E. Hoffstadt, of the Department of Bacteriology of the University of Washington, who tested it in the following manner.

0.1 cc. of the preparation was cultured on one plate each of 2 per cent plain agar, 2 per cent agar containing 1 per cent starch, and 2 per cent agar containing 10 per cent salt; these were incubated 72 hours, with controls, at a temperature of 8°C. Two other sets of plates of plain agar and starch agar, with controls, were incubated at 20 and 37°C. Finally, two anaerobic shakes in starch agar, sealed with vaseline, were incubated at 8 and 37°C. Growth in all cases was uniformly negative, indicating that our method of preparing the extract was not favorable to the survival of bacteria originally present in the digestive tract of *Bankia*.

We conclude therefore that the digestion of wood by *Bankia*, and in all probability by other ship-worms as well, is accomplished by a cellulase, which can be extracted and applied to the digestion of wood and cellulose under control conditions. It has been shown that the enzyme is not located in the crystalline style. It is, therefore, presumably resident in the so called livers or digestive diverticula attached to the stomach, Potts (1923) and Yonge (1926) having shown that wood particles are found, not only in the lumina

of these diverticula, but also in the interiors of the cells with which they are lined. Our investigations do not indicate whether the enzyme is extra- or intracellular in its operation.

SUMMARY.

1. A cellulase has been extracted from the anterior end of the digestive tract of the Northwest ship-worm (*Bankia setacea*), and successfully applied to the saccharification of both sawdust and filter paper.

2. Evidence is presented that the action is enzymic, and not bacterial.

3. It has been shown that the enzyme is not located in the crystalline style. It is therefore presumably resident in the so called livers or digestive diverticula attached to the stomach.

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THE FUNDAMENTAL FOOD REQUIREMENTS FOR THE GROWTH OF THE RAT.

II. THE EFFECT OF VARIATIONS IN THE PROPORTION AND QUALITY OF RECOGNIZED NUTRIENTS.*

By LEROY S. PALMER AND CORNELIA KENNEDY.

(*From the Section of Animal Nutrition, Division of Agricultural Biochemistry, University of Minnesota, St. Paul.*)

We have reported in a previous paper (1) the uniform experience of several years in which rats failed to grow normally on a presumably adequate diet of dextrin, highly purified casein, butter fat, salts, agar, and wheat embryo extract, when the animals were maintained on screens without access to bedding or excreta. In discussing this result it was stated that we have been unable to associate the subnormal growth with a deficiency of any of the definitely recognized nutrients. The present paper gives the results of our attempts to produce normal growth by varying the proportion and purity of the various ingredients in the basal diet, or through the addition to it of recognized nutrients, other than those known to be present.

Effects of Increasing Vitamin A and Adding Vitamins D and E.— Chart 1 shows the results of the various attempts to stimulate better growth by addition of more fat-soluble vitamins. The results show clearly that neither additional vitamin A + vitamin D as cod liver oil (Lots 3 to 8) nor vitamin D alone through irradiation[1] of the rats (Lot 9) or the diet (Lot 10) had a stimulating effect on growth.

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[1] The ultra-violet light for these tests was furnished by a Cooper Hewett Lab-Arc operating at 220 volts, 1 ampere. The rats received a 10 minute daily irradiation at a distance of 2 feet and each 500 gm. portion of the fat-free ration was given a single light treatment for 30 minutes at a distance of 18 inches.

CHART 1.

CHART 1. Lots 1 to 11. These rats show the results of modifying the basal Ration 5, so as to increase the percentage of vitamins A, D, and E. The basal ration consists of highly purified casein 18, salts (McCollum's Salt Mixture 185) 3.7, CaCO₃ 0.8, agar 2, butter fat 5, Crisco 10, alcohol extract of wheat embryo equivalent to 15 gm., ether-extracted embryo and dextrin to make up 100 parts. Lots 1 and 2 received 15 per cent butter fat and no Crisco. The rats in Lot 2 lost their hair in patches. The ration of Lots 3 and 4 contained 3 per cent cod liver oil and no other fat; the same type of ration replaced Ration 5 at the arrow for Lots 5 and 6, the cod liver oil being mixed in the diet for each day in the case of Lot 6. Lot 7 began with a ration containing 5 per cent butter fat, 5 per cent cod liver oil and 5 per cent Crisco. At point 1, the ration was changed to the basal Ration 5 and 1 gm. of butter fat fed separately daily, in addition. At point 2, the butter fat supplement was increased to 2 gm. daily. At point 3, the butter fat supplement was omitted and the basal ration modified to contain 5 per cent cod liver oil and 10 per cent Crisco, the butter fat being omitted. These rats became very greasy. Lot 9 received the basal Ration 5 and was irradiated for 10 minutes daily with ultra-violet light. These rats also became very greasy. For Lot 10, the ration itself was irradiated. Lot 11 received the basal Ration 5 to point 4 on the curves, when each rat was given daily 0.05 to 0.1 gm. of the non-saponifiable ether extract residue from wheat embryo oil. For the first 3 weeks the residue was fed dissolved in olive oil, but for the remainder of the experiment the crude residue itself was fed. The mean weekly food intake per rat for the several lots was as follows: Lot 1, 45 gm.; Lot 2, 29 gm.; Lot 3, 36 gm.; Lot 4, 35 gm.; Lot 5, 45 gm.; Lot 6, 29 gm.; Lot 7, 37 gm. until the butter fat supplement was fed when it increased to 47 gm.; Lot 8, 36 gm.; Lot 9, 32 gm.; Lot 10, 36 gm.; Lot 11, 35 gm.

Increasing the concentration of fat-soluble vitamins in the diet by increasing the proportion of butter fat from 5 to 15 per cent, omitting the Crisco, also failed to insure normal growth for Lots 1 and 2.   The butter fat used for Lot 1 represented fall pasture
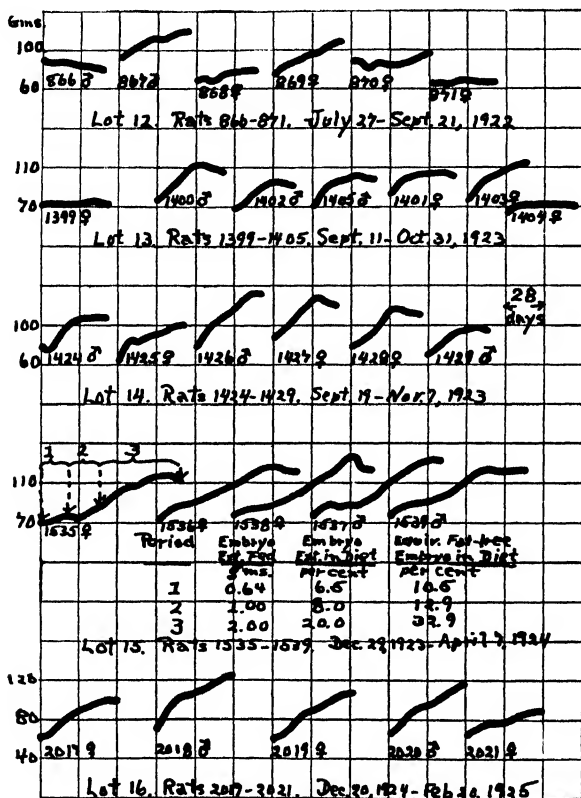
CHART 2. Lots 12 to 16.   These rats show the results of increasing the vitamin B-carrying portion of the basal Ration 5.   For Lot 12 the wheat embryo extract was increased from the equivalent of 15 to the equivalent of 25 per cent ether-extracted embryo.   Lots 13 and 14 had embryo extract at a level equivalent to 30 per cent ether-extracted material.   For Lot 15 the embryo extract was omitted from the basal ration and fed separately in quantities shown in the chart.   Lot 16 received the basal Ration 5 and a daily supplement of the Osborne and Wakeman yeast Fraction II, equivalent to 500 mg. of dry whole yeast.   The mean weekly food intake per rat for the several lots was as follows: Lot 12, 34 gm.; Lot 13, 34 gm.; Lot 14, 49 gm.; Lot 15, 45 gm.; Lot 16, 45 gm.

feeding, for Lot 2, a winter ration of grains, alfalfa hay, and corn silage. In the case of Lot 7, butter fat from flush summer pasture, fed separately after 7 weeks on a diet containing 5 per cent butter fat, 5 per cent cod liver oil, and 5 per cent Crisco, produced a temporary stimulation of growth of the male rats. This did not continue at the same rate, however, after the same butter fat was incorporated in the diet at a 15 per cent level.

There was indication that vitamin E has a slight growth-promoting effect in the case of Lot 11 fed 100 mg. daily of the crude unsaponifiable matter from wheat embryo oil (obtained by ether extraction) as a supplement to the usual basal ration. At first the vitamin E-containing material was fed suspended in olive oil, but later undiluted. In both cases the supplement was distasteful to the rats.

Effect of Increasing Concentration of Vitamin B.—Chart 2 shows the uniform failure of increasing the concentration of vitamin B in the basal diet. For Lots 12, 13, and 14, the wheat embryo extract was increased to the equivalent of 30 per cent of fat-free embryo in the diet. For Lot 15, the basal diet was free from vitamin B and the wheat embryo extract was administered separately in increasing amounts up to a maximum equivalent to 2 gm. of ether-extracted wheat embryo daily. No better results were secured in the case of Lot 16 where the basal ration, containing the equivalent of 15 per cent fat-free wheat embryo, was supplemented daily with the Osborne and Wakeman (2) yeast Fraction II equivalent to 0.5 gm. of dry starch-free yeast. The result is especially interesting, because we have found that 0.5 gm. daily of the whole yeast produces good growth when supplying the sole source of vitamin B for the basal diet. These results, together with others bearing on the relation of yeast and yeast fractions to growth with our basal diet, will be presented in another paper.

Effect of Increasing Both Vitamins A and B.—The rats in Lots 17 to 21, Chart 3, received the basal diet modified to contain the equivalent of 30 per cent wheat embryo, as was the case for Lots 12 to 16, Chart 2, and in addition, 15 per cent butter fat in place of 5 per cent used in the basal diet. The results of these tests present a very striking contrast to those previously reported. There is also a marked contrast between results of the four tests, although the diet was presumably identical. The rats of Lot 17
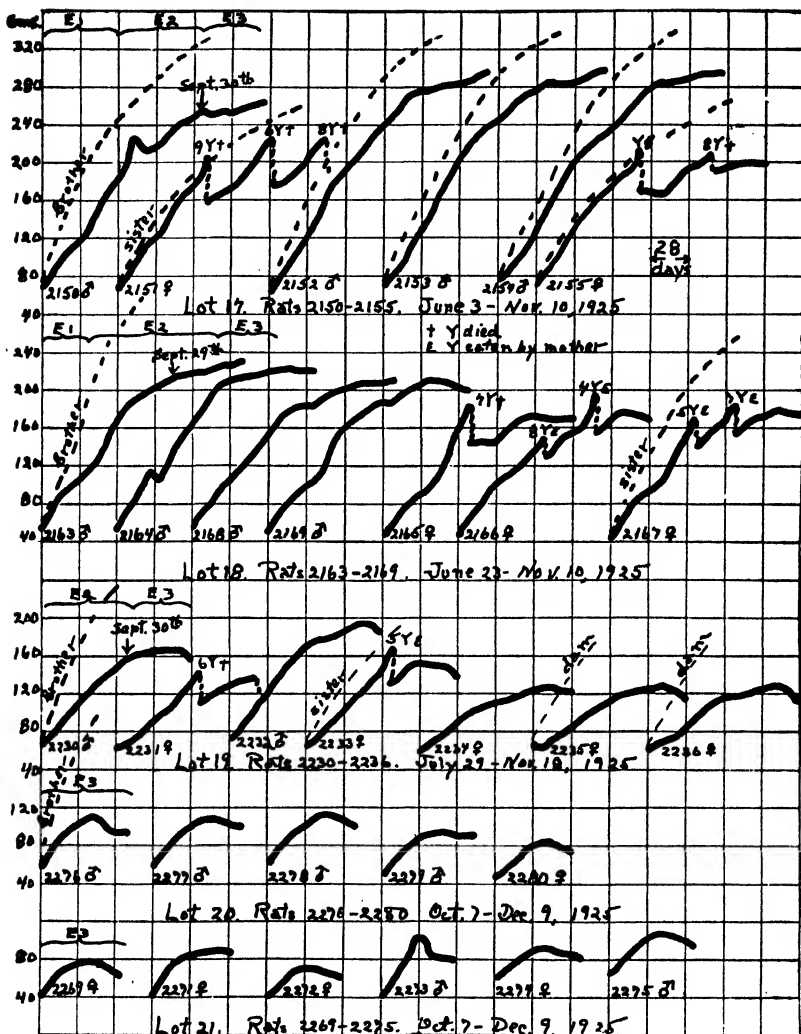
CHART 3. Lots 17 to 21. These rats show the effect of increasing both vitamin A and vitamin B in the basal Ration 5. For all lots the ration contained 15 per cent butter fat and no other fat, and the wheat embryo extract was increased to the equivalent of 30 per cent ether-extracted embryo. Growth became progressively poorer as the summer season advanced and with the use of different preparations of wheat embryo extract. Even the best growth fell considerably short of litter mate controls on the breeding stock diet. The range and mean weekly food intake per rat for the several lots was as follows: Lot 17, 51 to 99 (73) gm.; Lot 18, 40 to 73 (57) gm.; Lot 19, 25 to 72 (48) gm.; Lot 20, 28 to 53 (42) gm.; Lot 21, 21 to 47 (36) gm.

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were in good condition throughout the experiment. The rats of Lot 18 were thin, although their frames were large. Their fur was smooth at the close of the experiment but was very greasy from the 4th to 8th weeks. The rats in Lot 19 did not have a well nourished appearance at any time, and at the completion of the experiment were very thin, with rough greasy fur. Growth ceased entirely quite early in the case of Lots 20 and 21, with subsequent decline, the rats being in poor condition and greasy. Seven of the ten females in Lots 17, 18, and 19 bore young, several bearing two or more litters, but no young were reared.

In seeking the correct explanation of these results, it may be stated that the variation in results between the different lots in Chart 3 was not a question of lack of *inherited vigor*,[2] or of poor stock. The chart shows the excellent growth of brothers and sisters of the same or other matings when on our stock diet and housed on shavings. Lot 17, although making relatively better growth than Lots 18 and 19 in comparison with brother-sister controls, still came considerably short of attaining the rate of growth or maximum size of their litter mates. The rats of Lot 17 came from a litter of eleven, all reared and attaining a weight of 65 to 75 gm. at 28 days, when six were taken for experiment (Lot 17). The rats used for Lot 19 were put on experiment on the 27th day of age, with weights of 60 to 74 gm. The rats of Lot 20 were normal although not exceptional, going on experiment at 29 days of age with weights from 50 to 60 gm. The rats of Lot 21 were moderately good stock in the case of the larger group of litter mates (Rats 2269 to 2273), the remaining rats (Nos. 2274, 2275), also litter mates, being superior.

In comparing the progressively poorer growth of Lots 17 to 21, two facts stand out. The first is the decline in average food intake per rat per week, 73, 57, 48, 36 gm., respectively, for the five lots;

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[2] With the exception of two rats in Lot 19 (Rats 2235, 2236), and two rats in Lot 21 (Rats 2274, 2275), each lot represented a litter or portion of a litter. All the rats of Lots 17 to 20 were very closely related. The parents of Lot 17 were an outcross of rats inbred (brother and sister mating) for several generations, the dam coming from the same line as Lot 19. Lots 18, 19, and 20, were the result of brother-sister mating for four, five, and three generations, respectively, the parents of Lot 19 being brother and sister of the rats of Lot 18. The parents of Lot 20 had the same grandsire as the parents of Lot 19.

the second is that this decline in average food intake and the accompanying poor growth followed the advance in the season at which the experiment was begun, namely June 3, June 23, July 29, and October 7, two lots, Nos. 20 and 21, being started on this last date.   It will be noted, too, that the rats of Lots 17, 18, and 19 practically stopped growing before the rats in Lots 20 and 21 were started on experiment.   The date at which the weights became stationary is noted on Chart 3.

The failure of increased quantities of vitamin A alone (Chart 1 except Lot 7) or vitamin B alone (Chart 2) to stimulate growth whereas increasing both vitamins (Chart 3) gave much better growth, at least in the case of two out of five lots of rats, gives a first impression that the basal diet may be deficient in both vitamins A and B, and that the proportion of one or both of these growth factors approached adequacy only in the case of Lots 17 and 18.

Regarding vitamin A, butter fat is known to undergo seasonal variation in this factor.   However, the butter fat used was always prepared from fresh butter made from milk of the University herd.   Because of the superior feeding practice with this herd it is difficult to believe that 15 per cent of butter fat made from milk produced by it could ever furnish an insufficient amount of vitamin A.   Even in the case of Lots 20 and 21, there were never any signs of xerophthalmia or respiratory trouble in spite of the decline in weight noted on the chart.

In casting about for any other possible explanation which could be related to the butter fat we were impressed by a statement of Underhill and Mendel (3) that butter fat in their experience shows a seasonal variation in a factor, not identical with vitamin A, but effective in the cure of black tongue, the analog of pellagra in man.   This experience, although not duplicated by Goldberger and coworkers (4), raises a question whether we have encountered the same factor in the case of Lots 17 and 18, especially in view of Goldberger's experiments indicating the necessity of the antipellagra vitamin for growth in the rat.   Some support is given to this possibility in that the butter fat used in our experiments during the spring and summer of 1925 is seen to have had special growth-promoting properties in the case of Lot 7, Chart 1, in which some of the same butter fat was fed as a special supplement.

Regarding vitamin B, the method of preparation of the wheat embryo extract and the general uniformity of the commercial product we have employed make it unlikely that slight variations in concentration of vitamin B in different preparations of this factor could be of significance at the high level at which the product was fed; namely, the equivalent of 30 per cent. fat-free embryo. Nevertheless, there are some indications that a decline in the growth-promoting properties of the successive embryo extracts employed in the experiments of Lots 17 to 21, may have been responsible, in part, for the successively .poorer results secured. Three different preparations of wheat embryo extract were used, prepared from the same lot of commercial wheat germ.

The different periods during which different preparations of embryo extract were employed are shown on Chart 3 under the brackets, E1, E2, E3, above the first rat in each lot.   They appear to show that a marked decline in growth occurred during the use of preparation E2, and that practically no growth resulted when E3 was used.

Effect of Increasing Vitamin A and Wheat Embryo Extract and Adding Vitamin D.—The ration of Lots 22, 23, and 24, Charts 4, 5, and 6, was another modification of the basal diet.   The wheat embryo extract was increased to the equivalent of 30 per cent ether-extracted embryo as in the case of Lots 17 to 21.   In addition, the 5 per cent butter fat was replaced by 5 per cent fresh cod liver oil.[3]   The oil-free ration was prepared in kilo lots, but the complete diet containing the cod liver oil was prepared fresh each day in order to avoid any loss of vitamin A through oxidation of the oil kept in contact with the finely divided material.   The controls for Lot 22 were the parents; for Lots 23 and 24, litter mates of the experimental animals.

The results in these experiments were clearly superior to those obtained with the basal diet containing 5 per cent butter fat and wheat embryo extract equivalent to 15 per cent fat-free embryo, but the final result was the same.   The experiments with Lots 22 and 23 were divided into four periods, G1, G2, G3, and G4, representing four different preparations of wheat embryo extract.

[3] E. L. Patch Company Cod Liver Oil for Poultry and Animal Feeding, stated to restore growth of rats on vitamin A-deficient rations when fed in daily doses of 4 mg. or less.

The last three preparations were made from the same lot of wheat germ.   Preparation G2 was inadequate as was also preparation G4.   Although the growth rate was greatly accelerated when the change was made to preparation G3, the growth rate was not equal
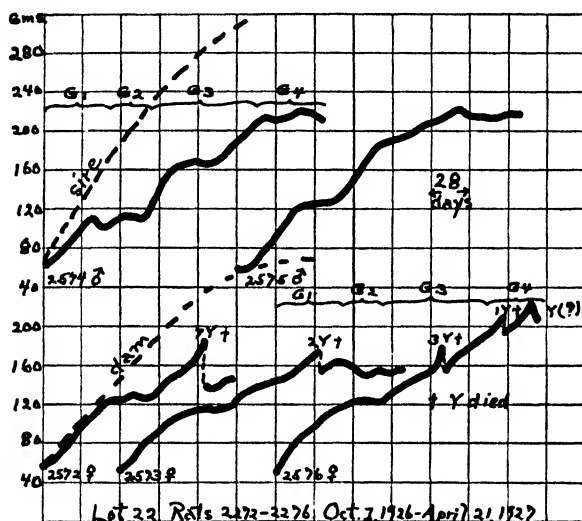
CHART 4.

CHARTS 4, 5, AND 6.   Lots 22, 23, 24.   These rats show the result of increasing the percentage of vitamins A and B and adding vitamin D to the basal Ration 5.   Wheat embryo extract was fed to each lot at a level equivalent to 30 per cent ether-extracted embryo.   The 5 per cent butter fat in the basal ration was replaced by 5 per cent cod liver oil, the oil being mixed into the daily ration of each lot.   Growth was fair but fell far short of parent or brother-sister controls on the breeding stock diet.   The different preparations of embryo extract used are noted on the chart under G1 to G5.   Extracts G1 to G4 were made by the same method from the same shipment of wheat embryo, Extract G5 from a new, fresh shipment.   The range and mean weekly food intake for the several lots was as follows: Lot 22, 28 to 64 (44) gm.; Lot 23, 27 to 82 (55) gm.; Lot 24, Rats 2641 to 2646, 29 to 52 (41) gm., Rats 2647 to 2651, 39 to 53 (45) gm.

to that of the controls of the same size except for a short period in the case of most of the rats.   Rats 2604 and 2605, Chart 5, were nearest to normal, but showed no tendency to catch up with their litter mate controls.   Instead there was a distinct tendency to fall away from the normal.   Lot 24, Chart 6, received the third
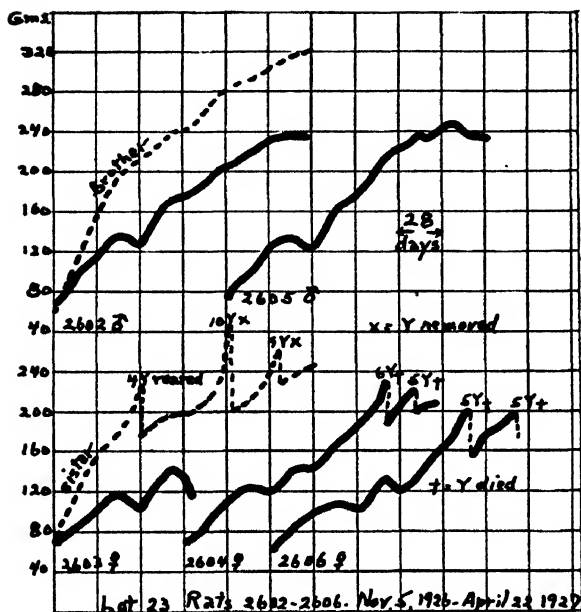
CHART 5.
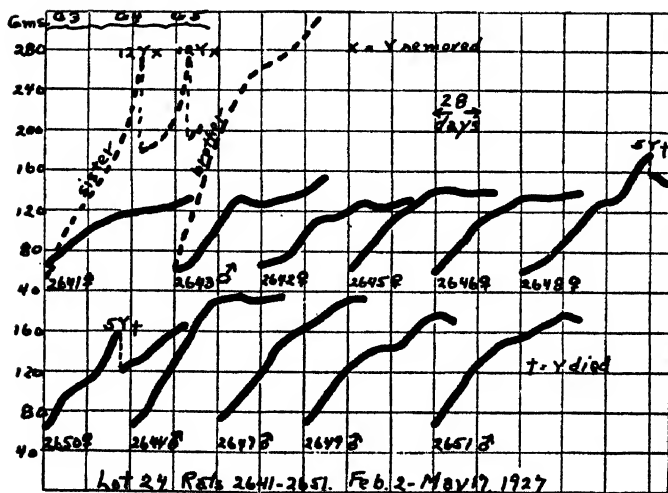
CHART 6.

preparation of embryo extract (G3) from the beginning of the experiment, but this was changed to the fourth preparation after 5 weeks, and 6 weeks later to a fifth preparation (G5) from a fresh shipment of wheat embryo.

The general results resemble those of Lots 17 to 21. Several litters were produced as in the previous experiments, but no young were reared. The rats of Lot 23 never had the normal sleek appearance of the properly nourished rat. It does not appear
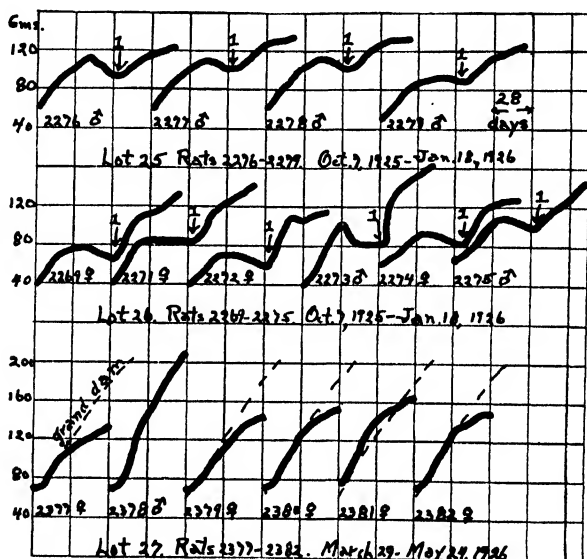
CHART 7. Lots 25 to 27. These rats show the effect of increasing vitamin A in the basal Ration 5, and giving excessive quantities of vitamin B. Lots 25 and 26 are the same as Lots 20 and 21, Chart 3. All of the 15 per cent fat in their ration was butter fat. The excessive vitamin B was begun at point 1 in the curves. For Lot 20 the vitamin B supplement was 500 mg. daily of yeast extract (Vegex) in addition to the wheat embryo extract in the basal Ration 5. For Lot 26 the wheat embryo extract or dextrin was increased to the maximum possible amount, replacing all of the carbohydrate; it amounted to the equivalent of 91 per cent fat-free embryo. Lot 27 received the same type of diet as Lot 26 from the beginning of the experiment. The embryo extract in the diet of this lot was likewise equivalent to 91 per cent of fat-free embryo. The range and mean weekly food intake per rat for the several lots was as follows: Lot 25, Period 1, 28 to 53 (42) gm., Period 2, 29 to 53 (38) gm.; Lot 26, Period 1, 21 to 47 (36) gm., Period 2, 49 to 67 (61) gm.; Lot 27, 40 to 66 (53) gm.

possible to attribute the ultimate failure in these experiments to a deficiency in any of the recognized nutrients. On the other hand there is a clear indication that the wheat embryo extracts employed varied in some growth-promoting quality in spite of the high level used.

Effect of Increasing Vitamin A with Excessive Amounts of Vitamin B.—Chart 7 shows the effect of large amounts of vitamin B-carrying substances when the basal diet contained 15 per cent butter fat. Lots 25 and 26 are the same as Lots 20 and 21, shown in Chart 3. The second portion of the curves shows the effect of further addition of vitamin B when superimposed on the diet already carrying alcohol extract equivalent to 30 per cent fat-free wheat embryo. Beginning at 1 on the curves, Lot 25 received 500 mg. daily per rat, of a brewers' yeast extract (commercially called Vegex), while for Lot 26, the embryo extract was increased to a maximum possible amount, equivalent to 91 per cent ether-extracted embryo. The ration of Lot 27 contained the equivalent of 90 per cent wheat embryo from the beginning of the experiment. The butter fat and embryo extract were of different sources for Lots 26 and 27, the butter fat being a fall and winter product for Lot 26, and an early spring product for Lot 27. The wheat embryo extracts, however, were prepared from the same lot of wheat embryo but represented different extractions.

The brewers' yeast extract fed to Lot 25 gave only a temporary stimulation to both appetite and growth. Excessive quantities of wheat embryo extract gave better results, indicating that small although insufficient quantities of some essential growth factor other than water-soluble B are carried by this preparation. One male rat in Lot 27 grew exceptionally well, but his litter mate sisters soon fell short of the growth attained by their ancestors. It may be pointed out also that the rats of Lot 27 were exceptional individuals, averaging 72 gm. at 28 days of age when placed on experiment. Their appetites remained good throughout the experiment but their external appearance was rather rough, and the abdomens and tails were badly stained a pronounced reddish color. We have noticed this discoloration of the fur rather frequently in rats on the basal ration as well as on various modifications of it, but have so far not arrived at a satisfactory explanation. It appears to be either a urine stain or due to erythrocytes.

A careful histological examination, kindly made by Dr. C. M. Jackson of the Institute of Anatomy, University of Minnesota, however, failed to reveal any lesions in the urinary tract (kidney, bladder, etc.).

Effect of Adding Vitamin C to Basal Diet.—Chart 8, Lot 28, shows the lack of effect of adding vitamin C to the basal diet. Each rat received daily 5 cc. of decitrated lemon juice which had been treated with Lloyd's reagent according to the method[4]
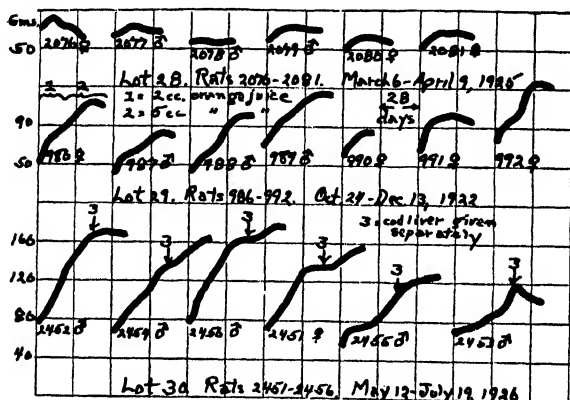
CHART 8. Lots 28 to 30. These rats show the results when vitamin C alone or vitamins A and B, or vitamins A, B, C, and D were added to the basal Ration 5. The basal ration contains no vitamin C. For Lot 28 the rats received a daily supplement of 5 cc. of decitrated lemon juice from which vitamin B had been removed by Lloyd's reagent (Harden and Zilva's method). For Lot 29 fresh, untreated orange juice was given daily in the amounts noted in the chart, adding both vitamins A and B. For Lot 30 the butter fat (5 per cent) in the basal diet was replaced by the same weight of cod liver oil and each rat given a daily supplement of 5 cc. of orange juice. At point 3 on the curves the cod liver oil was omitted from the diet and given separately, 0.5 cc. per rat daily. Four vitamins, A, B, C, and D, were thus added to the basal diet for this lot. The mean weekly food intake per rat in the several lots was as follows: Lot 28, 23 gm.; Lot 29, 43 gm.; Lot 30, 43 gm. The rats of Lot 29 became very greasy.

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[4] Lloyd's reagent was added to the fresh lemon juice at the rate of 5 to 10 gm. per 100 cc. and the mixture stirred vigorously with a mechanical stirrer for 1 hour. The earth and pulp were removed by suction filtration on a Buchner funnel, and the clear sparkling filtrate treated with $CaCO_3$, until effervescence ceased. The filtrate from the calcium citrate was used as the

of Harden and Zilva (5) for removing vitamin B from vitamin C-containing material.

Effect of Increasing Vitamins A and B and Adding Vitamin C to Basal Diet.—The effect on growth of augmenting the basal diet by additions of three vitamins, A, B, and C, is shown in Lot 29, Chart 8. This lot received fresh untreated orange juice as a daily supplement. For the first 2 weeks each rat received 2 cc. daily. For the remaining 4 weeks the dosage was 5 cc. Although the results were somewhat better than with Lot 28, they were in reality no better than has been secured in a number of instances with the basal diet alone.

Effect of Increasing Vitamins A and B and Adding Vitamins C and D to Basal Diet.—Lot 30, Chart 8, received fresh untreated orange juice and cod liver oil as the daily supplement to the basal diet, thus increasing the intake of four vitamins. The daily dosage of orange juice was 5 cc. per rat. For the first 6 weeks the basal diet contained 5 per cent cod liver oil in place of butter fat. The oil was omitted from the food mixture during the last 6 weeks, and the rats given the cod liver oil mixed with the orange juice, each rat receiving 0.5 cc. of oil daily.

The combination of recognized factors furnished by the cod liver oil-orange juice mixture caused apparently somewhat better growth than the best so far obtained with the basal diet alone. Failure eventually ensued, however, and the rats were quite greasy at the time the experiment was terminated.

Effect of Modification of Mineral Salts in Basal Diet.—Chart 9 shows the results of a limited study of varying the mineral salts. As pointed out in the first paper (1) of this series, the mineral salts were supplied in the early experiments by McCollum's Salt Mixture 185 (6). When the Johns Hopkins workers announced their results on calcium-phosphorus ratios and the optimum percentages of these elements required for the rat, calculation readily showed that the basal diet contained insufficient calcium to bring the percentage to 0.65. The correct percentage is obtained by adding 0.8 per cent $CaCO_3$ to the diet containing 3.7

source of vitamin C. Sufficient lemon juice was treated with Lloyd's re-. agent to furnish a week's supply; the decitrating process was performed daily.

per cent of Salt Mixture 185.   Lot 31, Chart 9, shows, however, that no benefit was derived from this change.   Nevertheless, we have continued to use the modification, in spite of the negative results obtained in this test.

For Lot 32 the basal diet was changed to include 4 per cent of Osborne and Mendel's (7) salt mixture in place of McCollum's No. 185.   This change was made to ascertain whether the deficiency in the basal diet could be caused by the lack of the less usual elements present in the Osborne and Mendel salt mixture, *i.e.* Mn, Al, F.   No benefit resulted from the change.
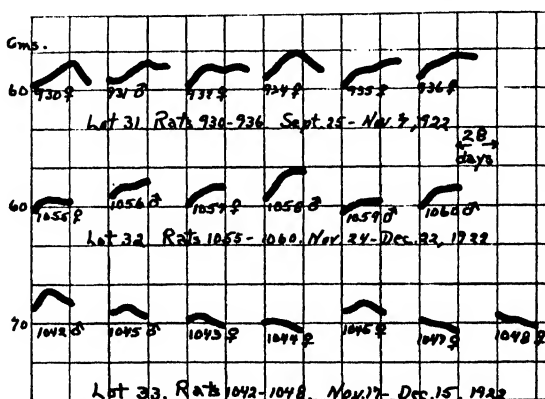
CHART 9. Lots 31 to 33.   This chart shows several modifications in the mineral portion of the diet.   Lot 31 had 0.8 per cent CaCO₃ added to increase the Ca to 0.64 per cent.   Lot 32 was given 4 per cent of Osborne and Mendel's salt mixture in place of McCollum's Salt Mixture 185.   Lot 33 received a double concentration of McCollum's salt mixture and 1.6 per cent CaCO₃.   The mean weekly food intake for the lots was as follows: Lot 31, 44 gm.; Lot 32, 35 gm.; Lot 33, 41 gm.   All the rats in these lots had rough, oily fur and were very thin.

In Lot 33 the usual salt mixture as well as the supplementary CaCO₃ was doubled.   The purpose was to determine whether a mineral salt content approaching that found in dry whole milk would overcome the growth failure.   It is obvious that insufficient minerals are not the cause of the growth failures on the basal diet.

Influence of Variations in Character of Carbohydrate Portion of Diet.—Chart 10 gives the results of varying the character of the

carbohydrate portion of the diet.   Lots 34 and 35 show a compari-
son of purified[5] and unpurified corn-starch dextrin.   In each case
the dextrin comprised about 50 per cent of the ration.   Both lots
failed miserably, but Lot 34, receiving the purified dextrin, de-
clined more rapidly.   The incorporation of the dextrin extracts in
the diet at the end of 4 weeks clearly stimulated both appetite and
growth.   The amount of extract added was equivalent to 100 per
cent dextrin.   These results indicate that corn-starch dextrin
contains traces of the growth-promoting substance lacking in
our basal diet.

Lots 36 and 37, Chart 10, received 25 per cent lactose in place
of an equal amount of dextrin in the basal diet.   For Lot 36, the
lactose was U.S.P. grade for infant feeding.   For Lot 37, the same
brand of lactose was recrystallized[6] twice from hot 70 per cent
ethyl alcohol.   It is evident from Chart 10 that lactose exerted
a profound effect on the nutritive value of the basal diet.   In
general, the results were decidedly in favor of the unpurified
lactose.   Growth was better, the rats never became oily, as did
Lot 37,[7] and one litter was successfully weaned, although the
young rats weighed only 20 to 22 gm. at 4 weeks.   The young
were, however, unable to survive on their mother's diet.

Chart 11, Lots 38 to 42, shows the results of further lactose
experiments carried out in 1926–27.   Litter mate or brother-sister
controls on the breeding colony diet were employed for all lots
except Lot 39.   25 per cent lactose replaced an equal amount of
dextrin in our basal Ration 5.   Lots 38, 39, and 40 received un-
purified U.S.P. lactose.   Lot 38 was fed lactose from the same
package used for Lots 36 and 37 in 1923, which had stood since

[5] The purified dextrin was prepared by continuous extraction for 5 days
with hot alcohol, using the Lloyd extractor, followed by a 48 hour ether
extraction in the same apparatus.   The dextrin was not dried following the
alcohol extraction, but was merely pressed.   The extracts were saved and
evaporated on some of the extracted dextrin.

[6] The lactose was dissolved in warm water to form a nearly saturated
solution, filtered, and the filtrate poured into sufficient 96 per cent alcohol
to give the desired strength of alcohol for recrystallizing.   The crystals
were filtered off by suction, washed on the funnel with 70 per cent alcohol,
dried, and the whole process repeated.

[7] One litter of Lot 36, partly raised, became very oily during the nursing
period.

that time in a sealed can in the laboratory.   The lactose used for
the other lots was presumably fresh.   It had the label of a differ-
ent manufacturer.   Lots 41 and 42 were fed the new lactose after
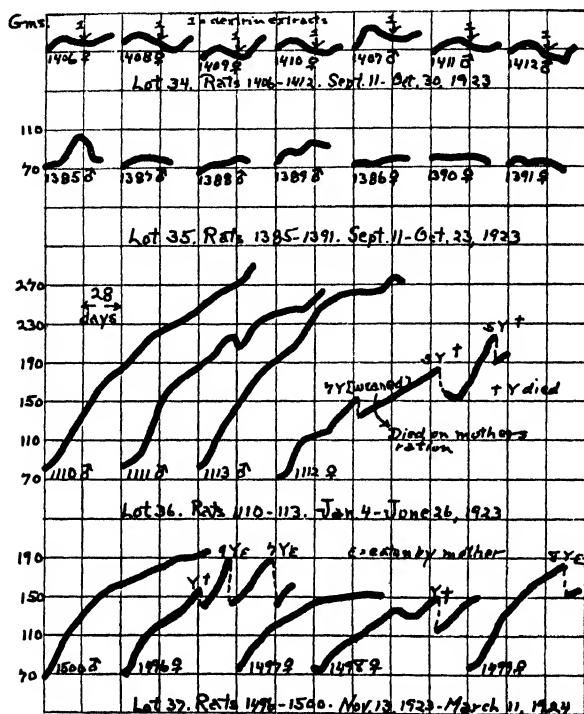
CHART 10. Lots 34 to 37.   The carbohydrate portion of Ration 5 was
modified for these rats.   Lot 34 received corn-starch dextrin which had
been thoroughly extracted with alcohol and ether in the Lloyd extractor.
The extracts were incorporated in the diet in amounts equivalent to 100 per
cent dextrin at point 1 on the curves.   The weekly food intake increased
from 14 to 38 gm. per rat and there was some increase in growth.   Lot 35
was a check lot receiving untreated corn-starch dextrin.   Usually our Ra-
tion 5 contains tapioca dextrin.   Lot 34 became very greasy but Lot 35
did not show this condition.   The rations of Lots 36 and 37 had 25 per cent
dextrin replaced by the same weight of u. s. p. lactose.   For Lot 37, how-
ever, the lactose was recrystallized twice from 80 per cent ethyl alcohol.
One litter reared by Rat 1112, Lot 36, died after weaning when placed on
the ration received by their mother.   The rats of Lot 37 became very thin
and their fur greasy during the last 2 months of the experiment.   The mean
weekly food intake of the several lots was as follows: Lot 34, 30 gm.; Lot
35, 30 gm.; Lot 36, 63 gm.; Lot 37, 63 gm.

extraction for 48 hours with U.S.P. ether in paper capsules in a Robertson extractor. Lot 39 received the ether-extracted lactose for the first 2 weeks of the experiment.

Two lots, namely Nos. 39 and 41, made some recovery from initial failure. Both untreated and ether-extracted lactose were represented. In the case of Lot 39, the measure of recovery noted did not immediately coincide with any change in the ration, but came 2 weeks after a change to a new preparation of wheat embryo extract (G3). The recovery noted in the case of Lot 41, coincided exactly with the change to the same vitamin B preparation. However, Lot 38, which received this preparation throughout the experiment, showed no such tendency. Lots 40 and 42, which received another wheat embryo preparation (G4) different from that of the other lots shown on Chart 11, made no appreciable growth. (See Charts 4, 5, and 6 for the effects of the same embryo preparations in other experiments.)

The possibility of other nutritive deficiencies being responsible for the poor growth in this group of experiments was given some study. Lot 40 was irradiated with ultra-violet light for 10 minutes daily for the last 2 weeks of the test, without effect. Fat from butter, canned in June, 1926, and kept in cold storage, was used for the first 18 days for Lot 41. No effect was noted.

The rats in Lots 39 to 41 became quite greasy shortly after the experiment started. This continued for several weeks in the case of Lot 39, and to the end of the experiment for Lot 40. Lot 41, which made the best growth in this group, never recovered a normal well kept appearance.

A comparison of the 1923 results with these more recent ones offers no satisfactory solution of the failure of Ration 5 to promote normal growth. The suggestion is offered that the older lactose contained some necessary factor, lost in long storage and absent from the lactose used in the recent work, but the evidence for this is admittedly circumstantial. The hypothesis of Emmett and Luros (8), that lactose and other unheated milk fractions carry a thermolabile water-soluble vitamin, necessary for growth, and distinct from vitamin B, may be invoked in support of the above suggestion, but the evidence available is insufficient to carry much weight. The well known effect of lactose on the intestinal bacterial flora which we were at first inclined to regard as a
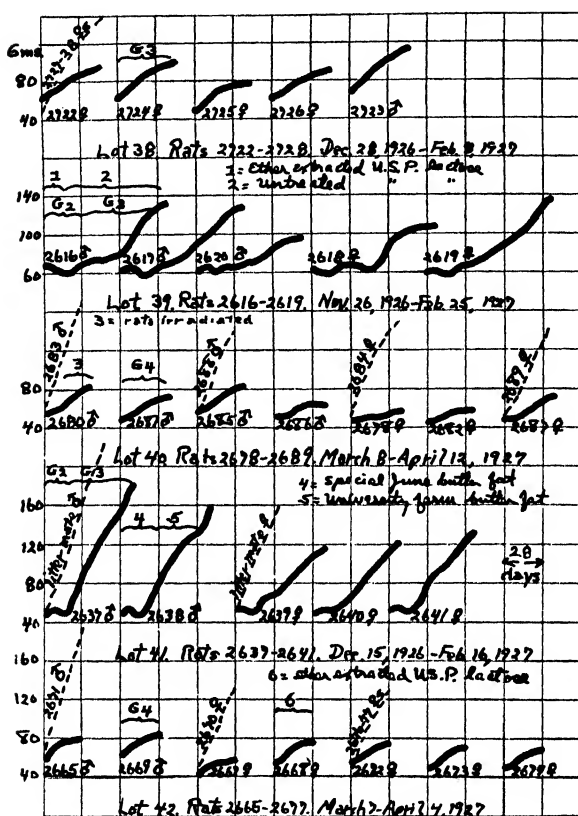
CHART 11. Lots 38 to 42. This chart shows a further study of the effect of incorporating 25 per cent lactose in the basal Ration 5, the experiments being carried out several years later than for Lots 36 and 37, Chart 10. The ration of Lot 38 contained the old lactose used for Lots 36 and 37 which had stood in the laboratory since the earlier experiments. Growth was poor but the rats did not become greasy. For Lot 39 new lactose, which had been thoroughly extracted with ether was used for the first 3 weeks when this was changed to untreated u. s. p. lactose. These rats became very greasy during the period of extracted lactose but this condition cleared up slowly when the untreated lactose was substituted. Lot 40 received the unextracted new lactose from the beginning. The rats of this lot became somewhat greasy. During the last 3 weeks of their trial, the rats received a 20 minute irradiation daily with the mercury vapor lamp. The diet of Lot 41 contained the new lactose, which had been thoroughly extracted with ether; in addition, butter fat was used which had been canned as butter in June, 1926, and kept in cold storage. It was a highly

possible explanation of the results of the 1923 experiments, has little support in the more recent series in which unpurified U.S.P. lactose proved to be of little or no benefit. We expect to study the problem further.

Effect of Increasing Protein Content of Basal Diet.—Chart 12, Lots 43 and 44, shows the results of increasing the casein to 50 per cent of the diet. The object of the experiment was to determine whether the purified casein carried small quantities of growth-promoting substance which would become manifest by increasing the concentration of casein. It is evident from the results that such is not the case.

Effect of Increasing Protein and Fat-Soluble Vitamins in Diet.— Lots 45 and 46, Chart 13, received increased proportions of casein and either vitamin A or both vitamins A and D. The diet of Lot 45 contained 50 per cent casein and 15 per cent butter fat in contrast with 18 per cent casein and 5 per cent butter fat in the basal diet. The diet of Lot 46 was similar except that the fat portion consisted of 12 per cent butter fat and 3 per cent cod liver oil. The butter fat for both lots came from cows fed grain, alfalfa hay, and corn silage. These variations had no marked effect on the usual unfavorable outcome, although the added cod liver oil in general seemed to cause a slight improvement when Lots 45 and 46 alone are contrasted.

Effect of Increasing Protein, Wheat Embryo Extract, and Fat-Soluble Vitamins.—Two experiments were conducted in which the casein was increased to 50 per cent of the diet and, in addition, the concentration of wheat embryo extract was doubled. In one of

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colored fat which gave a good color test for vitamin A, with the SbCl₃ method. This butter fat was used for 1 month only when the usual University Farm butter was substituted. The restored growth in this lot seemed to correspond with the change from wheat embryo Extract G2 to Extract G3. Lots 38 and 39 which received the same extract did not show this result. The rats of Lot 41 had rough, greasy fur throughout the entire experiment. This condition appeared within a week after the rats went on test. Lot 42 was another test of the ether-extracted lactose. These rats became somewhat greasy. Litter mate or brother-sister controls of previous litters on the breeding stock diet were used for most of the experiments shown on the chart. The mean weekly food intake per rat for the several lots was as follows: Lot 38, 33 gm.; Lot 39, 39 gm.; Lot 40, 32 gm.; Lot 41, 48 gm.; Lot 42, 35 gm.

the lots, vitamins A and D were added by replacing 3 per cent of the Crisco with 3 per cent cod liver oil. The two experiments are shown in Chart 14, Lots 47 and 48.

These modifications caused a noticeable improvement in the growth of the animals in comparison with the usual result with the
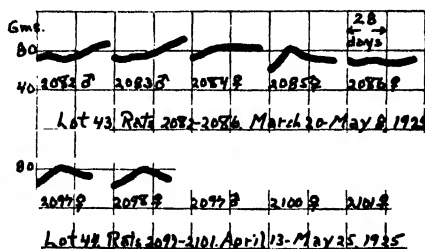
CHART 12. Lots 43 and 44. These lots received the basal ration modified to contain 50 per cent casein. Lot 43 received 10 drops of cod liver oil per rat daily for the last 2 weeks. Neither of these lots became greasy. The mean weekly food intake per rat was 34 gm. for Lot 43 and 31 gm. for Lot 44.
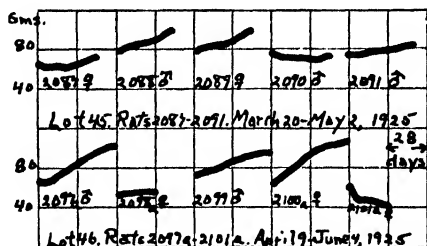
CHART 13. Lots 45 and 46. These rats received the basal Ration 5 modified to contain 50 per cent casein and a higher concentration of fat-soluble vitamins A and D. For Lot 45 this was accomplished by using 15 per cent butter fat in place of 5 per cent butter fat and 10 per cent Crisco. For Lot 46 the ration contained 12 per cent butter fat and 3 per cent cod liver oil. The same embryo extract was used for these two lots as for Lots 43 and 44. The mean weekly food intake per rat was 25 gm. for Lot 45 and 32 gm. for Lot 46. Lot 45 became slightly greasy but Lot 46 did not.

basal ration. The growth of the best rats, however, was far short of that of which the rats were capable, as can be seen by comparing the size of litter mates or parents on our stock diet. The improved growth was apparently especially true for Lot 48 receiving the additional vitamins A and D in the form of cod liver oil.
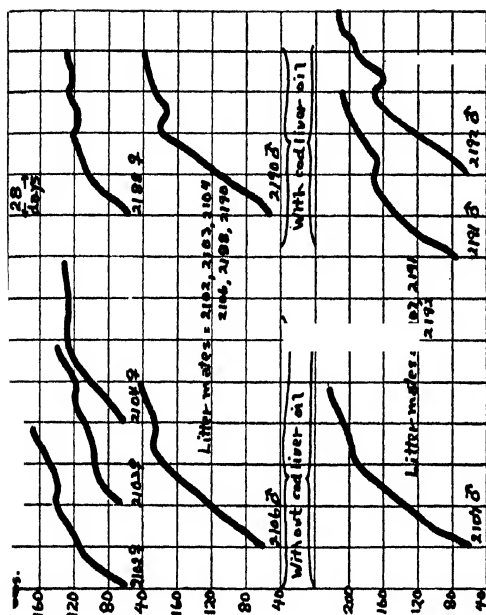
CHART 15.
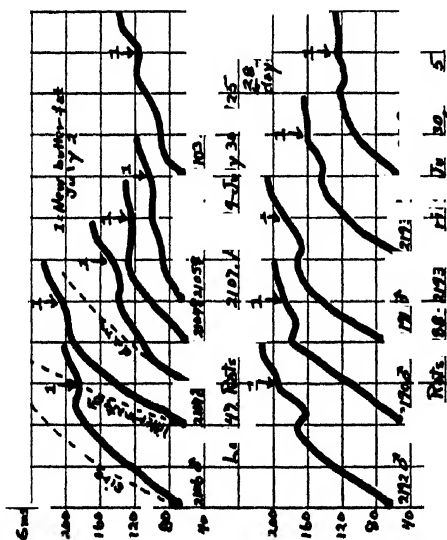
CHART 14.

CHARTS 14 AND 15. Lots 47 and 48. These charts show the result of concentrating the protein, vitamin B, and fat-soluble vitamins in the basal Ration 5. The ration of both lots contained 50 per cent casein, and wheat embryo extract equivalent to 30 per cent ether-extracted embryo. For Lot 47 the fats used were the usual 5 per cent butter fat and 10 per cent cod liver oil. Except Rat 2102, the rats in these two lots came from the two litters which were divided between the two lots. It appears from Chart 14 that Lot 48, receiving the cod liver oil, made the better growth but where litter mates are compared on Chart 15, it is seen that litter mates behaved alike and that the cod liver oil was without effect. When new butter fat, which had stimulated the growth of rats shown in Lot 7, Chart 1, and Lots 17, 18, and 19, Chart 3, was fed at point 1, growth was stimulated slightly for all rats. The rats of Lot 47 had a mean weekly food intake of 49 gm. per rat, and those of Lot 48, 48 gm. per rat. None of these rats became gr
ty but some of the rats in Lot 47 developed bald patches.

Two of the males in Lot 47 made nearly as good growth as the males in Lot 48, and one female, Rat 2193, in the same lot, made better growth than any of the other females. In order to explain these results, the growth curves are grouped in Chart 15 so as to show how the litter mates fared on the two rations. When this chart is inspected it is seen that the apparent differences due to the cod liver oil in the ration of Lot 48 disappear. Litter mates behaved alike.

The better growth obtained on these diets was thus due either to the combination of high protein and high vitamin B or to some
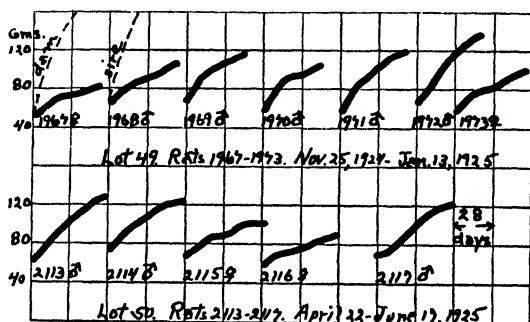
CHART 16. Lots 49 and 50. The proportion of all the ingredients in basal Ration 5 except the dextrin was doubled for these rats. All the rats became very greasy. This continued for 10 weeks beyond the curves shown in the case of Lot 49, and bald patches also developed. The animals grew slowly, two of the males attaining a weight of 200 gm. The maximum weight attained by any female was 150 gm. Lot 50, however, which showed the oiliness much more than Lot 49, gradually lost this condition, one male attaining a weight of 194 gm. during a further period of 10 weeks. The mean weekly food intake per rat was 39 gm. for Lot 49 and 40 gm. for Lot 50 during the growth period shown on the chart.

other factor. Inasmuch as neither high protein nor high vitamin B will alone insure the proper growth-promoting properties of the basal ration, one is confronted with the apparent fact that a combination of the two enhances the growth-promoting powers of the ration. This explanation is not particularly satisfying and seems paradoxical if not illogical. This is particularly true in view of the fact now rather definitely established (9) that increased proportions of protein require higher proportions of vitamin B.

This experiment, like that of Lot 7, Chart 1, and Lots 17, 18,

and 19, Chart 3, supports the view that butter fat, at times, may possess special growth-stimulating effects. When the same butter fat used for Lots 7, 17, 18, and 19 was fed to the rats in Lots 47 and 48, at point 1 on Chart 14, there was unmistakable evidence of a growth stimulation. When it is recalled that the diet of Lot 48 already contained 3 per cent cod liver oil, the effect of the fresh June butter cannot reasonably be attributed to vitamins A or D.

Effect of Increasing Concentration of All Essential Ingredients in Basal Ration.—Chart 16, Lots 49 and 50, shows the results of feeding a ration consisting of 36 parts casein, 20 parts Crisco, 22.6 parts dextrin, carrying wheat embryo extract equivalent to 30 per cent fat-free embryo, 10 parts butter fat, 7.4 parts salts (No. 185), 4 parts agar. The purpose of this modification was to determine whether a further concentration of the ingredients other than the strictly energy-yielding ones (except fat) would, with the same food ingestion as on the basal diet, bring about a sufficient increase in the intake of growth-promoting substances already present to modify the unfavorable outcome invariably obtained with the basal diet alone. The two groups of rats, Lots 49 and 50, were started on this diet 5 months apart, different lots of ingredients being employed. The uniformity in the outcome indicates a satisfactory constancy in the quality of the ingredients used for the basal ration.

It is evident that the somewhat more favorable growth obtained in contrast with the usual outcome indicates that the basal diet contains a certain, although inadequate concentration of all growth-promoting factors. The animals in Lots 49 and 50, however, fell far short of the growth rate they were capable of, as judged from the performance of their parents or litter mate controls on the breeding stock diet. The rats of both lots became very greasy, and those of Lot 49 developed bald patches.

Quality of the Protein Fraction as a Factor in Causing Deficiencies of Basal Diet.—Chart 17, Lots 51 to 55, shows the results of an attempt to attribute the poor growth to an amino acid deficiency in the diet. Lot 51 is typical of four lots, comprising twenty-five rats in all, fed cystine in addition to the basal diet. Lot 52 received 0.5 per cent cystine and, in addition, 1.8 per cent alcohol-soluble protein of milk. The latter supplement was used on the theory that our casein, free from alcohol-soluble protein because

of our method of purification, might require this fraction which is ordinarily mixed with it, in order to make the protein complete. The alcohol-soluble protein was prepared and purified by the method of Osborne and Wakeman (10).   There are no indications that the mixture of casein and alcohol-soluble protein benefited the animals.   In the case of Lot 53, the casein was fed at 25 per cent level without additional cystine, but 0.5 per cent alcohol-
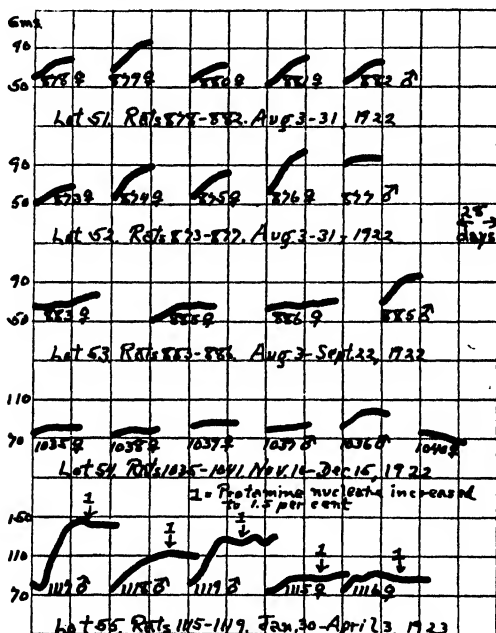
CHART 17. Lots 51 to 55.   An attempt was made with these rats to discover an amino acid deficiency in the basal Ration 5.   Lot 51 was given 0.5 per cent cystine in the diet, Lot 52, 0.5 per cent cystine and 1.8 per cent alcohol-soluble protein of milk, Lot 53, 25 per cent casein and 0.5 per cent alcohol-soluble protein of milk.   For Lot 54 the 18 per cent casein was replaced by 19.25 per cent protein mixture consisting of 84.4 per cent purified casein, 12.98 per cent heat-coagulable proteins of cow's milk (lactalbumin), and 2.62 per cent alcohol-soluble protein of milk.   Lot 55 was fed Ration 5 containing 0.5 per cent protamin nucleate.   This was increased to 1.5 per cent at point 1.   Lot 51 ate on the average 35 gm. of food per week per rat; Lot 52, 45 gm.; Lot 53, 41 gm.; Lot 54, 42 gm.; Lot 55, 45 gm.   For the first 4 weeks of the experiment with Lot 55, during which time the male rats grew fairly well, the average food intake of the lot was 54 gm.

soluble protein was added. The animals made practically no growth.

Lot 54 received a mixture of 16.25 per cent casein, and 2.5 per cent of the heat-coagulable portion[8] of milk whey. This was purified by the same procedure employed for the casein. Since lactalbumin, which undoubtedly comprised the bulk of the heat-coagulable material used, is known to possess an unusually high nutritive value, the growth failure of the rats in Lot 54 is further proof that our basal diet does not lack essential amino acids. Still further proof is seen in the results of Lot 55, which received protamin nucleate[9] as a supplement. Two male rats (Nos. 1117, 1119) grew well for about 30 to 35 days and then failed. An increase in the supplement to 1.5 per cent was without effect.

Effect of Varying Source of Protein in Basal Diet.—Four experiments were conducted with proteins other than casein in the basal diet. These are shown in Chart 18. Lot 56 was fed the basal ration containing 17 per cent purified[10] commercial egg white and 3 per cent water-washed fibrin. The fibrin was included in order to insure a cystine content of the diet equivalent to that when 18 per cent casein was used. The rats in this lot grew very well; in fact, practically duplicated the old Donaldson average. There was some reproduction, but no rearing of young. The rats themselves, however, never presented a normal appearance. The fur was always very scanty, and at the close of the experiment was oily and the hair falling badly, one rat (No. 1109a) being practically bald. The results were practically identical for Lot 57 fed a similar diet except that the commercial egg white was not extracted with alcohol and ether.

Lots 58 to 61, Chart 18, show a comparison of purified and unpurified muscle meat. The unpurified meat was fresh lean round of beef, which was boiled, ground, and dried. The purified

[8] This is frequently erroneously called lactalbumin although it is not a single protein when isolated by this method.

[9] This was a product of high purity prepared by Dr. Gortner of this Division, from salmon sperm according to the method given in Hoppe-Seyler, F., Handbuch der physiologisch- und pathologisch-chemischen Analyse, edited by Thierfelder, H., Berlin, 8th edition, 1909, 439.

[10] The egg white, composed of pale yellow scales, was extracted first with alcohol and then with ether in the Lloyd extractor, but it seems doubtful whether more than surface substances were extracted.
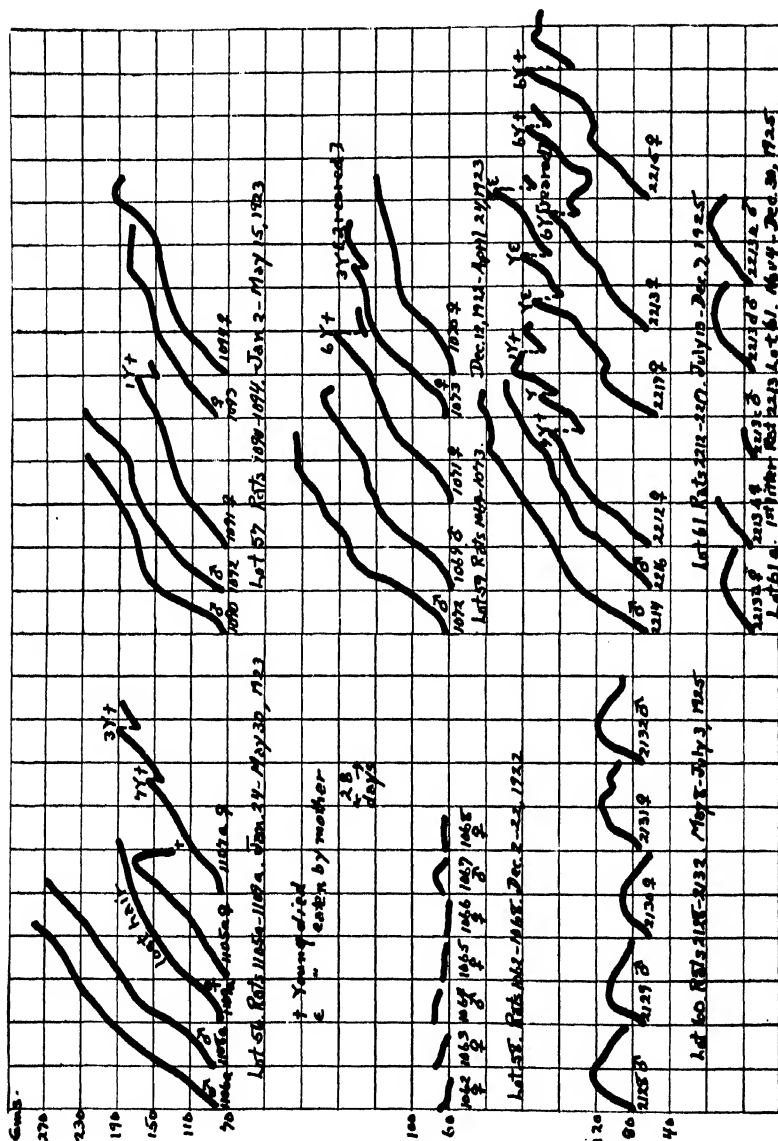
CHART 18.

CHART 18. Lots 56 to 61a. In these lots other proteins replaced casein in basal Ration 5. Lots 56 and 57 received a mixture of 17 parts of egg white and 3 parts of blood fibrin. The egg white and fibri re commercial products, of unknown origin. The egg white consisted of dry, pale yellow scales. For Lot 57 this pr was thoroughly extracted with 85 per cent alcohol, followed by ether, but very little extract was obtained. Each l rats ate on the average 65 gm. of ration per rat per week. All young born, died. Rat 1109a, Lot 56 became almost e ly nude and Rats 1092 and 1083, Lot 57, lost their hair in patches. Lots 58 and 59 show a comparison of purified an purified beef muscle meat. The protein material was prepared by the method of Osborne, Wakeman, and Ferry (1 This was used for Lot 59. For Lot 58 the moist material was carried through the alcohol and ether extractions in t oyd extractor, as we do for our purified casein. The ration of Lot 58 contained 18 per cent and that of Lot 59, 22 nt of dry meat substances. Rat 1073, Lot 59, reared two out of a litter of three young, but on the parents' diet the weighed only 42 gm. each when 48 days old. The mean weekly food intake per rat of Lot 58 was 54 gm.; of Lot gm. Lots 60 and 61 show another comparative test of purified and unpurified beef muscle meat, each at 18 per ce l. The appearance of Lot 61 was excellent throughout the entire experiment. Rat 2213 reared one litter of six your eir growth on the parents' diet is shown in Lot 61a. Of this litter, Rat 2213b, was killed by accident; Rat 22 as killed and eaten by its litter mates. The remaining rats became very greasy and lost a part of their fur. Th weekly food intake per rat was 39 gm. for Lot 60, 63 gm. for Lot 61, and 34 gm. for Lot 61.

meat was prepared according to the method of Osborne, Wakeman, and Ferry (11), and the still moist material carried through the alcohol-ether extraction in the Lloyd extractor as we do the casein in our method.   The ration of Lot 58 contained 23 per cent meat residue and Lot 59 received 22 per cent.   Lots 60 and 61 received only 18 per cent meat.   Lots 58 and 60 receiving the purified protein failed completely in spite of a good food intake in the case of Lot 58.   The better growth of Lot 60 at the beginning may have been due to more storage of essential growth factors. If so, the explanation lies in the improvement of the breeding colony ration during the 30 months interval between the two experiments, because the rats of Lot 58 were 6 weeks old when placed on experiment, while the rats of Lot 60, which made the better initial growth, were only 29 days old.

Growth was average for Lots 59 and 61 receiving untreated meat residue.   Reproduction occurred and a few young were successfully reared to weaning age.   Rat 1073 succeeded in rearing two of a litter of three, but they weighed only 42 gm. each at 7 weeks.   The one litter raised in Lot 61 was continued on the mother's diet.   The rats were small when weaned at 4 weeks (32 gm.), and while they grew slowly for a few weeks, they declined, and had to be chloroformed.   Their growth is shown on Chart 18 in connection with Lot 61.

Purity of Casein as a Factor in Failure of Basal Diet.—The results reported in Chart 18 offer presumptive evidence that the purification of the protein fraction of the basal diet is an important factor in causing the subnormal growth so invariably encountered.   We were strongly impressed with this possibility very early in our effort to determine the cause of the inadequacy of the diet.   Some of the information we have collected on this point from time to time is grouped together in Charts 19 and 20.

Lots 62 to 67, Chart 19, received commercial casein purified by the method, first employed by McCollum (12), of leaching in acid water for 7 days, the water being changed twice daily.   The origin, age, and method of manufacture of the casein used for Lots 62 to 65 is unknown, but it was probably a natural sour product, undoubtedly manufactured in the winter previous to its use by us in the spring and summer of 1923.   The casein used for Lots

66 and 67 was of local manufacture by the natural sour method in the winter of 1924–25. We believe that these facts, particularly the season of the year in which the casein was manufactured, are of importance in explaining the results with the acid-washed casein.

Lots 62 and 63 were given the Ration 5 formula. Lot 62 started to duplicate the usual results with the basal ration. The females were removed from the experiment after 1 month, but the males were continued. After 2 months they began to resume growth, one animal, Rat 1162, eventually attaining the older Donaldson average. This resumption of growth corresponds very closely with the period of maximum growth of Lot 63, started 1 month later than Lot 62, and suggests that some change in the quality of one of the other ingredients in the diet corresponding with the month of May was responsible both for the resumption of growth of Lot 62 and the best growth period of Lot 63. The two females of Lot 63 reproduced, one of them having three litters at regular intervals. No young were reared, however, all dying or being eaten by the mother.

The ration of Lot 64, started on experiment at the same time as Lot 63, contained the acid-washed, commercial casein also, and the equivalent of 30 per cent ether-extracted wheat embryo. The combination of (1) additional vitamin B, together with any other unrecognized growth factors in the embryo extract, and (2) the factor causing both the superior growth of Lot 63 and the resumption of growth of the two rats in Lot 62, produced exceptional growth in the rats of Lot 64, exceptional, at least, for experimental rats on screens, receiving a mixture of purified nutrients. Female 1230 reared its first litter which was continued on the same diet for 128 days, comprising Lot 65 shown on Chart 19. The second generation animals did well for about 2 months, but there was a rather sharp halt in their growth after 8 to 10 weeks.

Lot 67 was fed the same general type of diet as Lots 64 and 65, with acid-washed commercial casein and no vegetable fat. Growth was much better than for Lot 66 receiving the Ration 5 formula, although not as good as was obtained on the high embryo extract diet with Lot 63, 2 years earlier. The flattening of the growth curves in this experiment came in about 12 weeks. The feeding of 1 cc. of cod liver oil daily for 4 days beginning at point 1 on the curves for Lot 67, and the increase in butter fat from 5 to 15 per
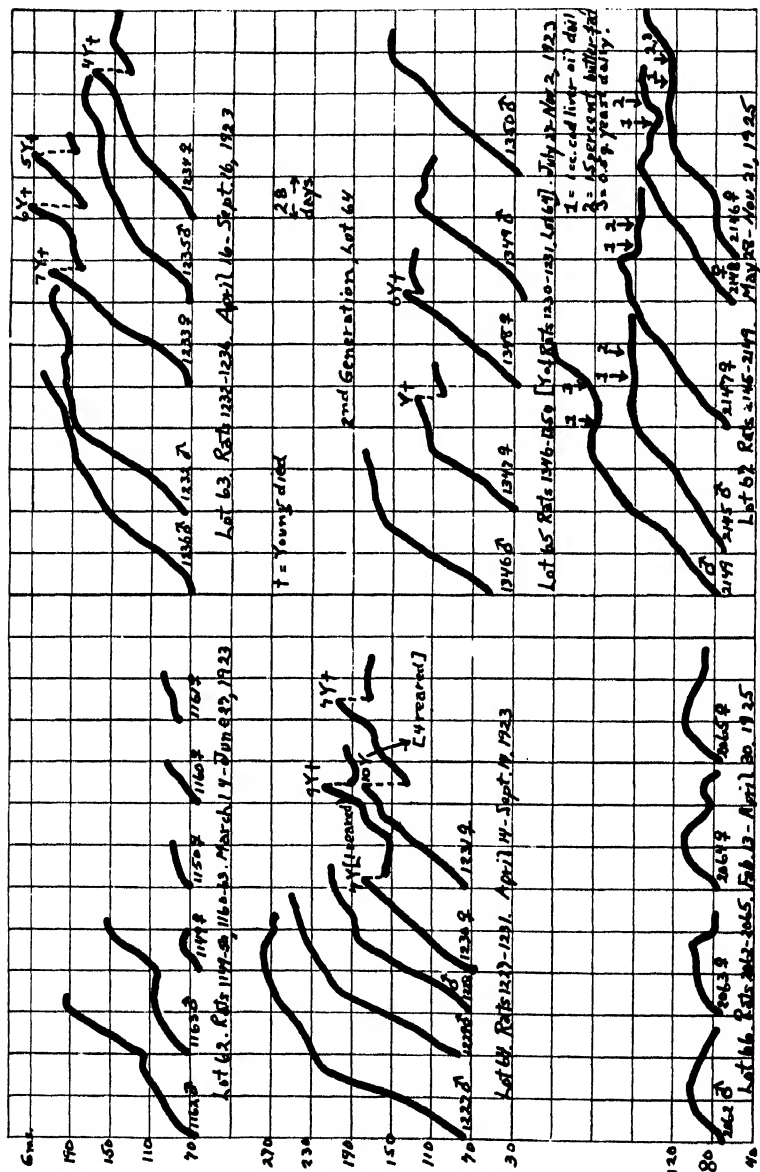
CHART 19.

CHART 19. Lots 62 to 67. This chart shows the results of using commercial casein washed in acid water instead of highly purified casein in the basal Ration 5, both with and without additional wheat embryo extract. Lots 62, 63, 66, and 67 received the usual proportion of wheat embryo extract equivalent to 15 per cent ether-extracted embryo, while Lots 64 and 65 received twice this proportion in their diet. Lot 65 was composed of the young reared by Rats 1230 and 1231, Lot 64. The renewed growth of the male rats in Lot 62 corresponded with a change in the wheat embryo extract used for Lots 63 and 64. Lot 66 received winter butter fat, Lot 67, spring and summer butter fat. Wheat embryo Extracts E2 and E3 (see Chart 3) were used for Lot 67. 0.5 gm. daily supplement of dry yeast was fed to Rats 2146 and 2149, beginning October 8. Vaginal smears made daily for the females of this lot for the last 10 weeks of the experiment showed irregular estrous cycles and two resorptions. None of the rats in any of the lots developed greasy fur. The mean weekly food intake per rat in the several lots was s follows: Lot 62, 48 gm.; Lot , 56 gm.; Lot 64, 61 gm.; Lot 65, 54 gm.; Lot 66, 36 gm. (53 gm. for 1st month); Lot 67, 43.
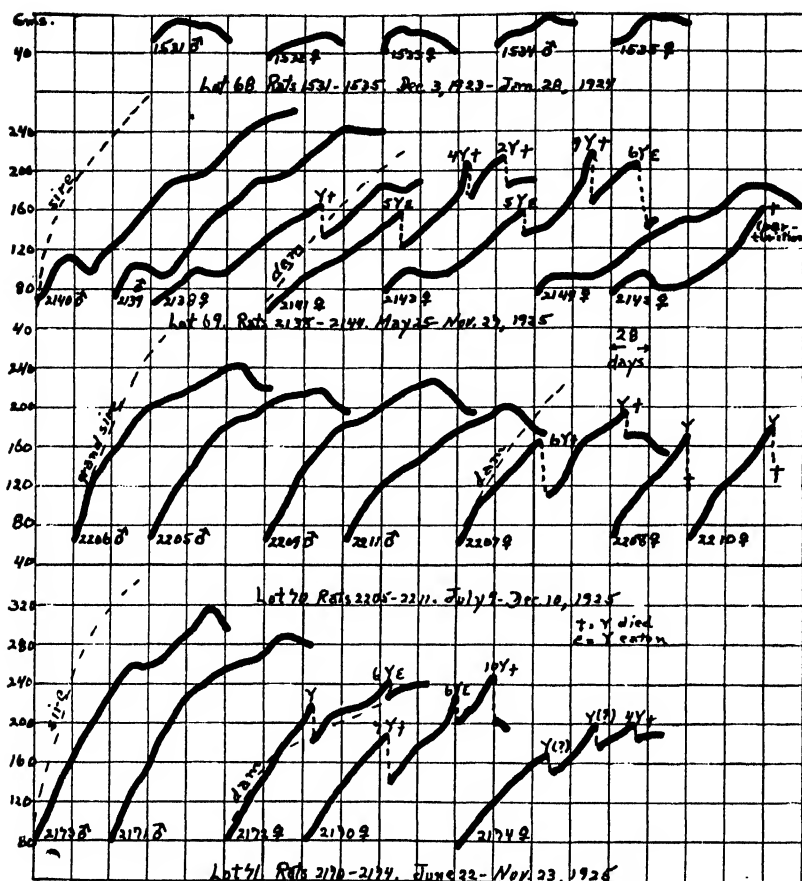
CHART 20. Lots 68 to 71. These rats show the effect on growth of the impurities carried by commercial casein when used in basal Ration 5.   Lot 68 received 20 per cent of a proprietary casein compound for infant feeding (Casec).   It was fed as purchased.   For Lot 69 unpurified winter, commercial casein was used together with 15 per cent butter fat in the diet (the Crisco being omitted).   The females in this lot showed regular estrous cycles, by the vaginal smear. Lot 70 received unpurified summer casein with 5 per cent butter fat and 10 per cent Crisco in the diet.   Lot 71 was fed the same casein as Lot 70 with 15 per cent butter fat and no Crisco in the diet.   The numerous litters in these lots all died or were eaten by the mothers.   Rat 2142, Lot 69, and Rats 2208, 2210, Lot 70, died during parturition.   The mean weekly food intake per rat was as follows: Lot 68, 44 gm.; Lot 69, 53 gm.; Lot 70, 55 gm.; Lot 71, 69 gm.

cent at point 2, was without effect and shows that vitamin A was not lacking. Rat 2146 given 0.5 gm. of dry yeast daily at this point, made a slight gain in growth, but evidently vitamin B deficiency is not involved or greater response would have resulted. The wheat embryo extracts employed for Lot 67 were the same as were used for Lots 17 to 21, Chart 3, where marked growth failure occurred at practically the same date as is noted in Chart 19, corresponding with a change to wheat embryo extract, E3.

Chart 20 gives the results secured in several trials in which commercial casein was used without further purification. Lot 68 received a proprietary calcium caseinate employed in preparation of protein milks for infant feeding. It was a white powder which readily dispersed in water to give a slightly alkaline fluid. The animals failed when this product constituted the protein of the basal diet at a level of 20 per cent. The wheat embryo extract for this lot was the same as was used with moderately successful growth for Lot 37.

Lots 69, 70, and 71, Chart 20, show an interesting variation in the nutritive value of the commercial casein. The diets of Lots 69 and 71 were exactly alike, except that the commercial casein employed for Lot 69 was made during the winter months while that used for Lot 71 was a product made in June. Both of these lots received 15 per cent butter fat, and no vegetable oil. The ration of Lot 70 contained the summer casein also, but only 5 per cent butter fat with 10 per cent Crisco.

Each of these three experiments, which ran simultaneously, showed a period of decline at the same time, apparently corresponding with some uniform change in the diets. All the animals were thin at the end of the experiment. The fur of Lot 70, especially, was rough. Disregarding the period of decline at the close of these experiments, the comparative results are strongly suggestive of a seasonal variation associated with the growth-stimulating properties of milk products, in this case both casein and butter fat. Lots 70 and 71, receiving summer casein, made better growth than Lot 69 which received winter casein, and of the two lots receiving summer casein, Lot 71 with 15 per cent butter fat in the ration was superior to Lot 70 with only 5 per cent butter fat. Undoubtedly, the same factor is involved adhering to or occluded in the casein in the one case, and dissolved in the butter fat in the other.

It is difficult to believe that the evidence as a whole points to the controlling factor being vitamin A or vitamin E.   This conclusion is supported by the normal estrous rhythm which was found to occur in the case of the females of Lot 69.   Daily vaginal smears were made for each animal beginning at point 1 on the curves until the close of the experiment except for the interval between the sign of pregnancy and occurrence of parturition.   Breeding did not occur at every heat stage, but there were only three cases of failure to impregnate after copulation, two of these being in the case of Female 2144 which did not litter throughout the experiment.   No resorptions were noted.

Two females in Lot 70, receiving summer casein and 5 per cent butter fat, died during parturition.   Neither the male nor female animals in this lot approached the size or growth rate of their ancestral stock shown in the broken lines.   The females of Lot 71, however, were practically as vigorous as their dam, but the males were far short of their sire, also shown in the broken lines.   Such differences as occurred between these two lots, as to growth, reproduction, physical condition, etc., would seem to be attributable solely to the difference in the butter fat content of the diet.

DISCUSSION.

We have presented in this paper some of our attempts to find a satisfactory solution of the failure of an apparently adequate diet of casein, salts (McCollum Salt Mixture 185), vitamins A and B, roughage (agar), and adequate energy to produce normal growth of rats when the animals are kept on screens to minimize coprophagy.   The experiments presented include the addition of vitamins A, B, C, D, and E, singly and in various combinations.   In other experiments qualitative and quantitative changes were made in the carbohydrate, protein, and mineral salts.   Practically every attempt to find the true cause of the growth abnormalities has been repeated with different lots of rats, in different seasons of the year and in different years.   The behavior of the animals in each lot has been as uniform as could be expected, but a lack of uniformity between different lots of animals has made a satisfactory solution of the problem increasingly difficult.   Variables, which we have so far been unable to control, evidently have been responsible, but it seems to us improbable that these variables are to be attributed to any of the definitely recognized nutrients.

Referring first to the vitamin experiments, we are convinced that a deficiency of vitamins C, D, and E is not responsible for the growth failures or subnormal growth encountered in these studies. We admit that the diet lacks vitamin C, but the results were decisive in showing that liberal portions of this vitamin were without effect. We also admit that the basal diet containing 5 per cent butter fat as the sources of vitamins A and D is quite low in vitamin D. However, we are forced to rule out vitamin D as responsible for the failures encountered because both cod liver oil and ultra-violet light when employed as the sole variable failed to modify the results. The potency of the cod liver oil used is also beyond question, both from the standpoint of the source of the oils used, and the methods employed in feeding it. Lack of vitamin E cannot be judged as the cause of the growth abnormalities in spite of the evidence that unsaponifiable residues from wheat germ oil stimulated growth slightly in our experiments. Inasmuch as vitamin E plays an important rôle in the development of the fetus, it should not be surprising that it is to be classified among the growth-promoting factors.

The evidence regarding a possible deficiency of vitamins A and B requires more careful study. Vitamin A deficiency alone is clearly not a factor because of the failure of vitamin A-rich cod liver oil to bring about sustained growth even when supplied to the animal separately each day. Some of the results at first sight seem to indicate simple vitamin B deficiency. For example, when the maximum possible quantity of wheat embryo extract was incorporated in the diet (equivalent to 90 per cent ether-extracted embryo) in the case of Lots 26 and 27, Chart 7, there was a marked stimulation to appetite and growth in the case of a majority of the animals. Against the view that this stimulation was due to vitamin B are the results with Lot 16, Chart 2, and Lot 25, Chart 7, in which additional vitamin B in the form of yeast extracts in 500 mg. daily doses produced either very poor growth or only a very temporary stimulation. Our experience with yeast and yeast fractions as supplements to the basal diet has been much more extensive than that given in this paper, and will be reported separately later. It will be sufficient to state at this time, that the general results support the conclusion that the effects secured with the large quantities of wheat embryo extract are not caused by the additional vitamin B, as such.

This conclusion is also supported by the variable results of the experiments reported in this paper in which wheat embryo extract equivalent to 30 per cent fat-free embryo was the source of water-soluble, growth-promoting vitamin (a 100 per cent increase over the basal diet, Ration 5), and in which either the fat-soluble vita-mins were also increased as butter fat or cod liver oil (Lots 17 to 24), or in which the casein was also increased from 18 to 50 per cent (Lots 47 and 48), or in which the casein employed was com-mercial casein, purified merely by acid washing (Lots 64 and 65). Although growth equal to controls on stock diet was never secured in these experiments, two lots (e.g., Nos. 17, 64) made growth com-parable to or better than the older Donaldson standard during the 6 months they were on experiment.   The growth of all the other lots receiving the double quantity of embryo extract was either subnormal or irregular or failed within a few weeks.   Records show that the growth failures or periods of no growth are corre-lated largely with variations in quality of wheat embryo extract. The other variations in the diet of this group are, therefore, of little importance and the two instances of good growth (Lots 17 and 64) are to be attributed to a high growth quality of the embryo extract.   This is likewise true of the periods of accelerated growth of Lots 22 and 23.

The particular problem we have been forced to decide in this group of experiments is whether the variations in wheat embryo extract are due to vitamin B or some other factor.   We have been reluctant to abandon the idea that it is vitamin B, but have been forced to do so because of the results with yeast extracts already mentioned.   .

In spite of the fact that variations in the wheat embryo extracts seem to be the important factor, we have also been impressed by the correlation between certain of the better growth records and the use of butter fat of early summer origin (Lot 17, first 3 months of Lot 18, Lots 47, 48, Lot 64).   One cannot help wondering what relationship exists between this correlation and the conclusion of Underhill and Mendel (3) that late spring and early summer butter fat is especially rich in antipellagra vitamin; a vitamin required for growth of rats according to Goldberger (4) and co-workers.   It is admitted that the periods of good growth found for Lots 22 and 23, which received cod liver oil in place of butter fat,

weakens the hypothesis, although it cannot be said to disprove it entirely. On the other hand, the excellent growth of Lot 36, Chart 10, may have been due in part to some such fat-soluble factor associated with the U.S.P. lactose which comprised 25 per cent of the ration, inasmuch as 5 per cent butter fat of winter origin (except for the last 6 weeks) was the only apparent source of fat-soluble vitamin and the equivalent proportion of wheat embryo the same as for the standard basal diet. The fact that Lot 37, receiving a similar diet except for a double recrystallization of the U.S.P. lactose from hot 70 per cent alcohol, made fair growth, seems to lend support to this view since any vitamins of the water-soluble type adhering to the U.S.P. lactose used for Lot 36 would be removed in the purification employed.

Unfortunately, a study of the possible occurrence of a necessary fat-soluble factor associated with commercial U.S.P. lactose carried out several years later with Lots 38 to 42 failed to establish the existence of any such factor in either lactose purchased at that time, or in some of the lactose which had remained in the laboratory since the earlier experiment with June butter fat. The rats in each lot behaved uniformly, but their growth varied with changes in wheat embryo extract rather than with the treatment of the lactose or the addition of June butter fat   The best growth observed, which was far short of the controls, was made with ether-extracted lactose and a certain lot of wheat embryo extract with a short period of June butter fat, but the same wheat embryo extract and old untreated U.S.P. lactose gave very poor results with another lot of rats.

Although the experiments with lactose in the basal Ration 5 as a whole fail to support the view that the presence of some unrecognized fat-soluble factor influenced the results in any of our experiments, and although the later group of lactose experiments was disappointingly poor, it is difficult to explain away the possibility that the lactose used for Lots 36 and 37, which grew normally or nearly so, carried some important growth factor, lost in storage and lacking from lactose purchased several years later. If the wheat embryo extract used for Lots 36 and 37 had been especially potent in growth-promoting power so that the results could be attributed solely to it, then Lot 55, which received the same extract as Lot 36 should not have failed. Likewise, Lot

15 which received the same embryo extract as Lot 37, in fact received it separately in more than double the concentration, also should not have failed so completely.

One other modification of the basal ration resulted in marked improvement in growth; namely, the substitution of the purified casein by impure casein or by other proteins which were impure or of questionable purity.   When the purified casein of the basal diet was replaced by commercial egg white plus fibrin, or dried muscle meat or commercial casein, the animals made much better growth, although not so good as controls on the breeding diet.   However, the purification of the muscle meat rendered it entirely incapable of supporting growth and the substitution of winter commercial casein by summer commercial casein greatly decreased the growth rate and size at maturity.   These results are not in themselves direct proof that unpurified proteins from animal products carry growth-promoting factors other than the recognized vitamins, but when considered in conjunction with our attempts to overcome the growth-promoting deficiencies of our basal diet by addition of the recognized vitamins, the results may well be interpreted as supporting some such view.

In concluding this discussion, we wish to emphasize the fact that the best growth attained in these studies has been considerably below the optimum possible on a diet of natural food.   We feel that until experimental animals on synthetic diets can be made to attain with regularity the growth possible on natural foods, our knowledge of the fundamentals for mere growth is still inadequate.   The question of reproduction, lactation, longevity, etc., when added to the growth question shows how large the field of endeavor is for these important biological problems.

CONCLUSIONS.

The fundamental food requirements for optimum or even average growth of the rat are not satisfied by the essentials at present universally accepted when the animal is housed so as to prevent as far as possible its coprophagistic habits.

The dietary deficiencies manifested under these conditions do not appear to be those of a mineral salt or amino acid nature, but are suggestive of vitamin-like factors.

It appears that vitamin A-carrying food substances and vitamin

B-carrying food substances or extracts vary in their growth-promoting qualities in a degree not explained by the vitamins of the water-soluble or fat-soluble groups at present generally accepted.

The purification of proteins of animal origin for use in synthetic diets may remove unrecognized dietary essentials for growth.

Impure commercial casein appears to undergo a seasonal variation in growth-promoting value not associated with recognized vitamins.

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STUDIES ON THE PHYSIOLOGY OF PYRIMIDINES.

By LEOPOLD R. CERECEDO.*

(From the Laboratory of Physiological Chemistry, Yale· University, New Haven.)

Recent studies on the chemistry of the pyrimidines have thrown much light upon the possible transformation which these substances may undergo in the course of their metabolism. Of these investigations that upon the oxidation of thymine has been particularly fruitful. Johnson and Baudisch (1) first observed that thymine is readily oxidized by ferrous bicarbonate and air, yielding products which are hydrolyzed to urea, acetol, and pyruvic acid. Deuel (2), in this laboratory, basing his experiments on these findings, observed that thymine and uracil, when fed to dogs, increased the urinary urea output, thus showing that the oxidation of these pyrimidines *in vivo* seems to follow the same paths it does *in vitro*. Wilson (3) observed a large increase in the elimination of urea after feeding uracil nucleoside to rabbits. Baudisch and Bass (4) studied the action of hydrogen peroxide, and of hydrogen peroxide and ferrous sulfate on thymine. They found that the same products were formed as in the oxidation with ferrous bicarbonate and air. Baudisch and Davidson (5) investigated the mechanism of the oxidation of thymine (I), and were able to show that 4,5-dihydroxyhydrothymine (thymine glycol) (II) is an intermediary product in this reaction.

$$
\begin{array}{cccc}
\text{NH—CO} & & \text{NH—CO} & \\
| \quad | & & | \quad | \diagup \text{CH}_3 & \\
\text{CO} \quad \text{CCH}_3 & \rightarrow & \text{CO} \quad \text{C} & \\
| \quad || & & | \quad | \diagdown \text{OH} & \\
\text{NH—CH} & & \text{NH—CHOH} & \\
\end{array}
$$

I. Thymine.   II. Thymine glycol.
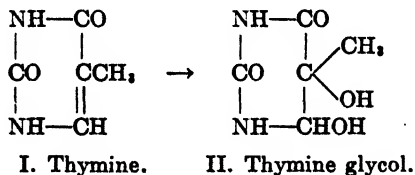
The only investigation on the metabolism of cytosine recorded in the literature was carried out by Mendel and Myers (6). They showed that in the organisms of the rabbit, dog, and man cytosine was excreted unchanged. Liver extract was found to have no marked effect on it.

The behavior of the pyrimidines towards microorganisms has recently been studied by Hahn and his coworkers. Hahn and Lintzel (7) observed that living yeast cells in the presence of sucrose, and cell-free yeast extracts deaminize cytosine at room temperature with formation of uracil. Thymine and uracil are not affected by yeast or by yeast extracts. Hahn and Schäfer (8) investigated the action of *Bacillus coli* on uracil, thymine, and cytosine. They found that only the latter is attacked, being deaminized to uracil, while thymine and uracil remain unchanged.

Of great interest has been the report of Johnson and Coghill (9) on the isolation of 5-methylcytosine from the hydrolytic products of tuberculinic acid. The relationship between 5-methylcytosine (III) and thymine (IV) is shown as follows:

$$
\begin{array}{cc}
\mathrm{N} = \mathrm{CNH_2} & \mathrm{NH-CO} \\
| \quad\quad | & | \quad\quad | \\
\mathrm{CO} \quad \mathrm{CCH_3} \;\;\rightarrow\;\; & \mathrm{CO} \quad \mathrm{CCH_3} \\
| \quad\quad \| & | \quad\quad \| \\
\mathrm{NH-CH} & \mathrm{NH-CH}
\end{array}
$$

III. 5-Methylcytosine.   IV. Thymine.
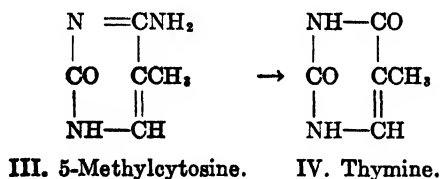
Hahn and Haarmann (10) have shown that, under the action of yeast extract, 5-methylcytosine is deaminized and converted into thymine.

On perusal of the literature referred to above, several questions presented themselves for investigation. The interesting studies of Baudisch and Davidson on the mechanism of the oxidation of thymine, suggested the idea of investigating whether, as in the case of thymine *in vitro*, thymine glycol is an intermediate oxidation product *in vivo*. In view of the findings of Hahn and his collaborators as regards the action of yeast extracts and *Bacillus coli* on cytosine, it seemed desirable to study anew the metabolism of this pyrimidine. Lastly, we decided to determine whether 5-methylcytosine is metabolized in the animal body in the same fashion as the other pyrimidines.

EXPERIMENTAL.

Dogs were used exclusively in the experiments described below. The animals were kept on the standard diet described by Cowgill (11). The plan of the experiments was to follow the urinary nitrogen, urea, ammonia, and inorganic sulfur, until a nitrogen equilibrium had been reached, to give the substance to be tested either separately by mouth or mixed with the food, then to continue the experiment until the output of these excretory products had returned to normal. The animals were kept in metabolism cages, and the urine collected by catheterization every 24 hours. They were allowed to drink water *ad libitum*.

The following methods for analysis were used: total N, Kjeldahl; urea and ammonia N, Van Slyke and Cullen; inorganic sulfur, Folin. The pyrimidines were isolated from the urine by the procedure described by Mendel and Myers (6).

Previous investigators have fed uracil and thymine in form of their sodium salts. Inasmuch as these salts are strongly alkaline in aqueous solution, we eliminated any possibility of alkali formation by feeding these pyrimidines as such mixed with the food.

Experiments with Uracil and Thymine.

In Tables I and II are recorded the results obtained with uracil and thymine. In the first experiment (Table I) 3.0 gm. of uracil mixed with the food were given to Dog A. The urine of the next day showed a marked increase in urea. No uracil could be recovered from the urine of the experimental period.

Table II shows the results of the experiment with thymine. In this experiment we also found a distinct increase in the output of urea. To meet any possible objection that the increase in urinary urea may have been due to an increased endogenous metabolism in the animal, caused by the ingestion of the pyrimidines, the output of inorganic sulfur as an index of protein catabolism was determined in these and the following experiments. Folin (12) and Shaffer (13) have shown that the inorganic sulfur in the urine runs parallel with urea. An examination of Tables I and II will demonstrate that on the days of increased urea excretion the output of inorganic sulfur remained practically constant.

Experiments with Thymine Glycol.

The purpose of the following experiments was to determine whether thymine glycol is an intermediary metabolic product of thymine.

TABLE I.

Uracil.

Dog A.   Weight 13.0 kilos.

Date.	Volume.	Total N.	Urea N.	NH₃ N	Sulfur.
Apr. 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
3–4	197	7.82	6.23	0.59	0.445
4–5	165	7.86	5.94	0.59	0.436
5–6	177	7.81	6.00	0.63	0.426
6–7	175	7.96	5.97	0.64	0.459*
7–8	200	8.77	6.50	0.63	0.456
8–9	150	7.97	5.98	0.61	0.466
9–10	200	8.43	6.22	0.69	0.479

* 3.0 gm. of uracil fed with food.   N = 0.75 gm.

TABLE II.

Thymine.

Dog A.   Weight 13.7 kilos.

Day.	Volume.	Total N.	Urea N.	NH₃ N	Sulfur.
1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
May 29–30	192	6.69	5.23	0.46	0.355
" 30–31	185	6.84	5.36	0.43	0.342
June 1–2	155	6.70	5.22	0.39	0.362*
" 2–3	137	7.34	5.56	0.44	0.364
" 3–4	177	7.11	5.40	0.47	0.348
" 4–5	165	7.23	5.32	0.41	0.356

* 2.5 gm. of thymine fed with food.   N = 0.55 gm.

In the first experiment (Table III) 2.0 gm. of thymine glycol were given mixed with the food to Dog C.   In Table IV are presented the data obtained after feeding 2.5 gm. of thymine glycol to Dog D.   In both experiments we find an increase of urea in the urine after ingestion of this pyrimidine.   These

results seem to indicate that thymine glycol may be one of the intermediate stages in the metabolism of thymine.

On account of the instability of thymine glycol in alkaline solution no attempts were made to recover the substance from the urine.

TABLE III.

Thymine Glycol.

Dog C.   Weight 10.72 kilos.

Day.	Volume.	Total N.	Urea N.	NH₂ N	Sulfur.
June, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
18–19	145	5.71	4.54	0.31	0.297
19–20	140	5.86	4.61	0.32	0.299
20–21	145	5.83	4.57	0.36	0.316*
21–22	141	6.11	4.68	0.36	0.308
22–23	140	5.76	4.44	0.37	0.303
23–24	140	5.83	4.50	0.47	0.318
24–25	130	5.82	4.57	0.42	0.303

* 2.0 gm. of thymine glycol fed with food.   N = 0.35 gm.

TABLE IV.

Thymine Glycol.

Dog D.   Weight 7.85 kilos.

Day.	Volume.	Total N.	Urea N.	NH₂ N	Sulfur.
June, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
23–24	110	4.65	3.62	0.26	0.223
24–25	110	4.76	3.63	0.24	0.223
25–26	125	4.57	3.55	0.25	0.234*
26–27	115	5.11	3.82	0.27	0.227
27–28	125	4.77	3.57	0.23	0.241
28–29	115	4.63	3.61	0.22	0.234
29–30	95	4.55	3.53	0.26	0.232

* 2.5 gm. of thymine glycol in 50 cc. of solution.   N = 0.44 gm.

Experiments with Cytosine.

Mendel and Myers (6) reported only one experiment on the metabolism of cytosine in the dog.   They injected 2.0 gm. of cytosine hydrochloride and recovered 0.215 gm. from the urine unchanged.

In our first experiment (Table V) 2.5 gm. of cytosine mixed with the food were given to Dog C. An examination of the data will show that the ingestion of this pyrimidine did not alter the output of urea. The protocol demonstrates an increase of urea on the 2nd day after the administration of the cytosine. This was not due to the cytosine, as the higher output of inorganic sulfur on the same day shows. From the urine of the experimental period it was possible to recover 0.69 gm. of uracil. Analysis (Kjeldahl nitrogen) gave 24.56 per cent of nitrogen instead of 25.00 per cent. From the same urine 0.42 gm. of cytosine was isolated in form of the picrate.

TABLE V.

Cytosine.

Dog C. Weight 7.0 kilos.

Day.	Volume.	Total N.	Urea N.	NH$_2$ N	Sulfur.
June, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
10–11	125	5.60	4.39	0.36	0.279
11–12	120	5.63	4.41	0.32	0.284
12–13	120	5.63	4.39	0.37	0.280*
13–14	190	6.23	4.34	0.29	0.273
14–15	150	6.14	4.68	0.39	0.310
15–16	140	5.73	4.32	0.30	0.299
16–17	145	5.71	4.54	0.31	0.297

* 2.5 gm. of cytosine fed with food.   N = 0.95 gm.

Deuel (2) has shown that uracil and thymine, when fed to dogs in relatively large amounts, are partly excreted unchanged; if fed in small amounts, however, they are completely metabolized. It seemed of interest to determine how cytosine would behave under the same conditions.

In Table VI are recorded the results of one experiment in which 0.5 gm. of cytosine was given daily to Dog C over a period of 6 days. It was possible to isolate from the urine 1.22 gm. of cytosine picrate, equivalent to 0.4 gm. of cytosine. The small amount of cytosine recovered seems to justify the conclusion that cytosine, under the conditions of this experiment, was more completely metabolized than in the experiment reported above (Table V).

Table VII presents the data obtained after feeding 3.0 gm. of

cytosine hydrochloride to Dog B. No marked increase in the output of urea was observed. From the urine of the experimental period 0.8 gm. of uracil was recovered. Analysis by Kjeldahl

TABLE VI.

Cytosine.

Dog C. Weight 10.72 kilos.

Day.	Volume.	Total N.	Urea N.	NH₃ N	Sulfur.
June, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
22–23	140	5.76	4.44	0.37	0.303
23–24	140	5.83	4.50	0.47	0.318
24–25	130	5.82	4.57 ·	0.42	0.303*
25–26	150	6.21	4.51	0.39	0.310*
26–27	125	5.68	4.22	0.31	0.297*
27–28	150	5.89	4.20	0.36	0.301*
28–29	140	5.98	4.36	0.41	0.306*
29–30	140	5.96	4.55	0.37	0.305*
June 30–July 1	150	5.99	4.50	0.41	0.307
July 1– " 2	115	5.87	4.46	0.38	0.315
" 2– " 3	200	6.09	4.58	0.39	0.323

* 0.5 gm. of cytosine mixed with the food. N = 0.19 gm.

TABLE VII.

Cytosine.

Dog B. Weight 9.34 kilos.

¡Day.	Volume.	Total N.	Urea N.	NH₃ N	Sulfur.
July, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
10–11	105	5.03	3.79	0.22	0.224
11–12	100	5.02	3.80	0.27	0.222
12–13	110	5.05	3.94	0.30	0.237
13–14	110	4.97	3.92	0.31	0.232*
14–15	305	5.72	3.62	0.33	0.209
15–16	142	4.80	3.51	0.39	0.219
16–17	145	5.07	3.97	0.29	0.225

* 3.0 gm. of cytosine hydrochloride in 75 cc. of water *per os.* N = 0.85 gm.

analysis yielded 24.42 per cent of N instead of 25.00 per cent. From the same urine 0.31 gm. of cytosine was isolated in form of the picrate.

Experiments with 5-Methylcytosine.[1]

In Tables VIII and IX are shown the results obtained after feeding 5-methylcytosine. An examination of the data will demonstrate that the administration of this pyrimidine did not

TABLE VIII.
5-Methylcytosine.

Dog B.   Weight 9.4 kilos.

Day.	Volume.	Total N.	Urea N.	NH₃ N	Sulfur.
May, 1927	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*
23–24	140	4.70	3.51	0.37	0.238
24–25	120	4.74	3.63	0.31	0.234
25–26	165	4.89	3.77	0.32	0.233*
26–27	257	5.25	3.52	0.35	0.208
27–28	105	4.41	3.40	0.30	0.206
28–29	130	4.60	3.60	0.28	0.218
29–30	150	4.87	3.75	0.31	0.242

* 2.5 gm. of 5-methylcytosine hydrochloride in 100 cc. of water *per os.*
N = 0.65 gm.

TABLE IX.
5-Methylcytosine.

Dog B.   Weight 9.3 kilos.

Day.	Volume.	Total N.	Urea N.	NH₃ N
July, 1927	*cc.*	*gm.*	*gm.*	*gm.*
18–19	140	5.21	3.95	0.304
19–20	135	5.19	3.99	0.316
20–21	165	5.09	3.93	0.290*
21–22	270	5.73	3.85	0.289
22–23	145	4.81	3.54	0.279
23–24	140	5.03	3.89	0.293

* 3.0 gm. of 5-methylcytosine HCl in 100 cc. of H_2O *per os.*   N = 0.78 gm.

cause an increase in the output of urea or ammonia. From the urine of the first experiment (Table VIII) it was possible to isolate 2.7 gm. of 5-methylcytosine picrate. In the urine of the second experiment 1.05 gm. of thymine were excreted. The

[1] I am indebted to Dr. H. H. Harkins for the 5-methylcytosine used in these experiments.

analysis by the Kjeldahl method gave 21.03 per cent of N instead of 22.22 per cent. From the same urine it was also possible to recover 1.72 gm. of 5-methylcytosine picrate, equivalent to 0.6 gm. of 5-methylcytosine.

DISCUSSION.

The results of the experiments with thymine and uracil confirm those obtained by Deuel to the effect that these pyrimidines, when fed in physiological amounts to dogs, are metabolized to urea. As regards the fate of the 3-carbon atom chain of the molecule we know nothing at the present .time.

In the experiments with thymine glycol evidence was obtained indicating that this substance may be an intermediary metabolite of thymine. This is another example of the analogy between many types of tissue oxidation and those effected by hydrogen peroxide. Furthermore, these results seem significant in connection with the question of uricolytic destruction of uric acid in man. They suggest that compounds of a similar structure may occur as intermediate oxidation products of uric acid in human tissues. Uric acid glycols have been described by Behrend and by Biltz in their investigations on the oxidation of uric acid. Recent experiments reported by Schittenhelm and Chrometzka (14) seem to support the above expressed view. They found that allantoin administered orally to a human subject was partly excreted unchanged, partly destroyed, leading to an increased output of urea.

Contrary to our expectations the experiments with cytosine show a different behavior of this pyrimidine in metabolic processes. We are reminded of the resistance of the amino-purines to enzymes as compared with the oxypurines. From the work of Jones, Schittenhelm, and others, it appears that the amino-purines are less resistant to deaminizing enzymes when in organic combination than in the free state. Our results show a close analogy between cytosine and the amino-purines in this respect.

SUMMARY.

Experiments are described in which uracil, thymine, 4,5-dihydroxyhydrothymine, cytosine, and 5-methylcytosine were fed to dogs maintained on a nitrogen equilibrium.

Evidence was obtained that uracil and thymine, when fed in small amounts to dogs, are metabolized to yield urea.

The results obtained with 4,5-dihydroxyhydrothymine make it seem probable that this substance is an intermediate metabolic product of thymine.

When cytosine and 5-methylcytosine are fed to dogs, they are partly excreted unchanged, partly deaminized to uracil and thymine respectively.

Further experiments on the metabolism of pyrimidines are in progress.

I wish to express my sincere thanks to Professor Lafayette B. Mendel for suggesting this research, and for much valuable advice and criticism.

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SOME EFFECTS OF SYNTHALIN ON METABOLISM.

By N. R. BLATHERWICK, MELVILLE SAHYUN, and
ELSIE HILL.

*(From the Chemical Laboratory of the Potter Metabolic Clinic, Santa
Barbara Cottage Hospital, Santa Barbara.)*

Last year, Frank, Nothmann, and Wagner (1) reported the synthesis of a guanidine compound having an insulin-like effect upon the normal and diabetic organism. Unlike insulin, this substance causes a lowering of the blood sugar when taken by mouth. In "synthalin" the hypoglycemic power of the parent substance, guanidine, has been so greatly increased that the investigators mentioned have felt justified in recommending the use of this compound in the treatment of diabetes. They recognized the inherent toxicity of the substance and prescribed certain regulations to be followed in using it. These consist of a gradual increase in dose up to a certain point and the introduction of a day's rest between periods.

Lewis and his collaborator, Izume (2, 3), found that the hypoglycemia produced by hydrazine is accompanied by an increased amount of the amino acids in the blood. They showed, further, that there was a failure to deaminize glycine when injected. These and other observations led them to consider that the hypoglycemia due to hydrazine is produced as follows: "It is suggested that the primary cause is failure of normal glyconeogenesis, the transformation of non-carbohydrate materials to glucose, as a result of which the supply of glucose available is diminished because of the hepatic injury produced by hydrazine." It occurred to us that the action of synthalin might resemble that of hydrazine. We have, therefore, conducted experiments much like those referred to in connection with hydrazine.

Methods.

Rabbits which had fasted for 24 hours were used. These animals had been used previously for insulin testing and some had received various compounds of lead. We do not believe that the former treatments had any bearing upon the results of the present investigation. The methods of Folin and Wu were used for the estimation of sugar, urea N, and amino acid N of the blood. The synthalin was the product manufactured by Kahlbaum. Each tablet contained 25 mg. When it was desired to give the substance by injection, the tablets were ground in a mortar, dissolved in enough water to give a concentration of 2.5 mg. per cc., and then the insoluble coating was filtered out. The filtrate gave an immediate guanidine reaction with the diacetyl reagent. For the oral experiments the entire tablets dissolved and suspended in water were given by stomach tube. The glycine injected was dissolved in either water or 0.7 per cent NaCl solution. Glucose in 20 per cent solution was administered by stomach tube at the rate of 1.75 gm. per kilo of body weight in the tolerance experiments.

Oral Administration.

Three rabbits were given 2.0, 7.6, and 33 mg. of synthalin per kilo without evidence of hypoglycemia during periods of 6 to 9 hours following the doses. Another rabbit weighing 3.0 kilos was given 25 mg. a day for 3 days, fasted 1 day, and then given 25 mg. the following morning. Determinations at 1.5, 3, 4.5, 6.25, and 23 hours after this last dose showed no significant change in blood sugar. Still another rabbit being fed a diet of alfalfa hay and crushed barley was given 25 mg. of synthalin on May 25, 27, 28, and 30, and on June 1. The sugar, urea N, and amino acid N of the blood were followed at intervals from May 25 to June 4. There was no significant change in either the sugar or the amino acid N. The urea showed an initial value of 25.1 mg. of N per 100 cc. and tended toward a lower value until on June 4 duplicate determinations revealed a content of 11.8 mg. per 100 cc. During this period the animal lost in weight from 3.65 to 3.25 kilos. It may be noted here that the normal value for the urea N

of rabbit blood lies between 20 and 30 mg. per 100 cc. Our experiments with the oral administration of synthalin failed to show any effect upon the blood sugar content. The decreasing values for urea N and the loss in body weight are indicative of a toxic action. The rabbit either fails to absorb the hypoglycemic principle or so alters it as to make it inactive.

Parenteral Administration.

In marked contrast are the effects produced when synthalin is given either subcutaneously or intravenously. Hypoglycemia is nearly always obtained. The rapidity and extent of the hypoglycemia appear to be dependent upon the amount of the substance injected. The maximum dose which allows recovery of the rabbit is about 4 mg. per kilo. This applies to both forms of administration. The convulsions are not entirely like those produced by insulin. The animals kick somewhat, especially with the hind feet, become rigid, and die. The heart continues to beat for some minutes after respiration has ceased. The blood is dark in color. Administration of glucose is much less effective in reviving the animals than in the case of insulin. The changes in blood sugar following the subcutaneous and intravenous injection of synthalin are recorded in Table I.

It will be noted that all of the rabbits showed a hypoglycemia following the administration of 4 mg. or more per kilo excepting Rabbit C. This animal also had no decrease in blood sugar at a later trial. Rabbit V responded with a marked hyperglycemia followed by a hypoglycemia terminating in death.

Interesting and important changes in the urea and amino acid N accompany the changes in blood sugar. See Table II.

The outstanding feature of the experiment with Rabbit W is the marked accumulation of urea. This is an invariable result when the animal survives a sufficient length of time. Shorter experiments such as that with Rabbit V show a beginning retention of urea. The urine of such animals contains protein. Further experiments show that this is an acute nephritis from which the rabbits recover in 3 or 4 days. Probably not less significant is the increase in amino acids shown by both Rabbits W and V. It is not always possible to demonstrate this latter phenomenon. Experiments such as these led us to determine whether synthalin

TABLE I.

Blood Sugar Following Injection of Synthalin.

Rabbit No.	Weight.	Synthalin injected.	Form of administration.	Blood sugar in mg. per 100 cc.														Remarks.
				Initial.	1 hr.	1.5 hrs.	2 hrs.	2.5 hrs.	3 hrs.	4 hrs.	4.5 hrs.	5 hrs.	6 hrs.	6.5 hrs.	8 hrs.	9 hrs.	9.5 hrs.	
	kg.	mg. per kg.																
B	1.80	14.0	Subcutaneous.	100	105													
X	3.00	10.0	"	105		95	74	30*	38	27*								Sugar, 50 mg. at 25 hrs.; died.
37	3.40	5.0	"	100	110				111		110			87	75	65		
C	3.10	4.0	"	118						148			133					No hypoglycemia from 12 to 20 hrs.
V	2.50	2.5	"	95														
K	3.60	8.0	Intravenous.	95		61		29*										Sugar, 93 mg. at 24 hrs.; died 36 hrs. after injection.
W	2.30	5.0	"	93		80			54			83	55	95				
V	2.60	5.0	"	91		167			220					*				
B	2.00	4.0	"	103		87			62			69				114		

* Died.

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decreases the ability of an animal to deaminize subcutaneously injected glycine.

Influence of Synthalin on Amino Acid Tolerance.

The plan of experimentation was as follows: Rabbits were given subcutaneous injections of glycine amounting to 1 gm. per kilo

TABLE II.

Blood Changes Following Injection of Synthalin.

Rabbit No.	Date.	Synthalin injected.	Blood content.			Remarks.
			Sugar.	Urea nitrogen.	Amino acid nitrogen.	
	1927	*mg. per kg.*	*mg.*	*mg.*	*mg.*	
W	May 3		93		8.2	
	9.00 a.m.	5 intravenously.				
	10.30 "		80	33.3	8.1	
	12.05 p.m.		54	33.3	8.7	
	3.00 "		83	46.7	10.2	
	May 4					
	9.30 a.m.		93	125.0	18.0	
	May 5					
	10.50 a.m.		111	192.3	8.5	Died during night of May 5–6.
V	May 11		91	19.2	10.1	
	9.10 a.m.	5 intravenously.				
	10.40 "		167	19.3	10.1	
	12.10 p.m.		220	20.4	9.9	
	3.05 "		55	22.7	11.1	
	4.15 "					Convulsions.
	4.20 "			27.8	17.9	Died. Blood from aorta.

of body weight. Estimations of amino acid N, urea N, and sugar in the blood were made at intervals up to 24 hours. The animals were allowed to recuperate for a period of about 3 weeks. They were then given 4 mg. of synthalin per kilo intravenously and immediately following that 1 gm. of glycine per kilo subcutane-

TABLE III.

Blood Changes Following Injection of Glycine Alone and With Synthalin.

Date.	Rabbit No.	Substance.	Amount in 100 cc. blood.						Remarks.
			Initial.	1.5 hrs.	3 hrs.	6 hrs.	10 hrs.	24 hrs.	
1927			*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
May 26	109	Urea N.	26.0	27.8	32.5	32.9	27.8	24.0	*
		Amino "	8.5	25.7	22.2	14.3	9.1	8.6	
		Sugar.	100	105	105	129	100	105	
June 17		Urea N.	31.4	29.9	30.3	35.7	49.0	75.8	†
		Amino "	9.6	31.1	30.4	29.5	20.0	15.7	
		Sugar.	98	75	77	65	83	91	
May 23	118	Urea N.	36.5	42.9	45.3	56.7	67.2	57.1	*
		Amino "	8.8	27.2	17.9	11.9	9.3	8.6	
		Sugar.	108	114	105	121	105	105	
June 6		Urea N.	54.9	58.1	56.2	65.4	65.8	83.3	† Died in convulsions 55 hrs. after injections. Urea N, 131.2; amino N, 17.5; sugar, 50.
		Amino "	10.0	28.6	27.2	22.4	23.1	16.7	
		Sugar.	95	68	55	89	114	85	
June 2	A	Urea N.	28.1	29.9	32.7	37.9	38.5	32.9	*
		Amino "	7.0	21.4	16.4	8.5	7.9	5.2	
		Sugar.	100	105	100	118	100	95	

Date	No.								Notes
June 22		Urea N.	24.5	24.3	28.2	38.8	55.5	133.3	† Died 2nd night following injections.
		Amino "	6.9	23.0	23.5	23.3	20.0	11.8	
		Sugar.	100	91	74	45	69	80	
June 14	31	Urea N.	27.0	28.7	29.2	39.1	35.3	31.7	*
		Amino "	10.1	25.7	25.5	16.1	11.3	8.8	
		Sugar.	114	121	125	121	121	118	
June 15	C	Urea N.	24.4	29.2	34.7	33.6	51.6	71.4	† 72 hrs. after injections. Urea N, 76.9; amino N, 8.2; sugar, 108.
		Amino "	9.7	27.7	31.5	25.2	20.7	8.5	
		Sugar.	108	111	105	103	114	108	
July 1	U	Urea N.	45.0	57.5	61.8	96.5	100.0	142.5	* Given 4.7 mg. uranium nitrate per kilo. Died night of July 3.
		Amino "	7.9	24.2	20.0	13.0	9.5	7.5	
		Sugar.	100	114	111	108	100	105	
June 20	44	Urea N.	16.2	15.9	19.2	20.3	21.7	21.5	1.5 units insulin per kilo intravenously and 1 gm. glycine per kilo subcutaneously at same time.
		Amino "	7.3	25.4	17.5	7.2	6.5	6.3	
		Sugar.	119	65	60	100	103	100	

* Glycine, 1 gm. per kilo subcutaneously.
† Glycine, 1 gm. per kilo subcutaneously; synthalin, 4 mg. per kilo intravenously.

ously. The same determinations were made as before. The results are recorded in Table III.

When glycine alone was injected, the amino acids of the blood returned to the initial level at from 6 to 10 hours after the injection; the urea increased and then returned to the normal level; and there was a tendency to a mild hyperglycemia at about 6 hours after the injection. The findings agree well with those of Lewis and Izume, excepting that they observed no increase in blood sugar after the injection of this amount of glycine.

The administration of synthalin and glycine at the same time
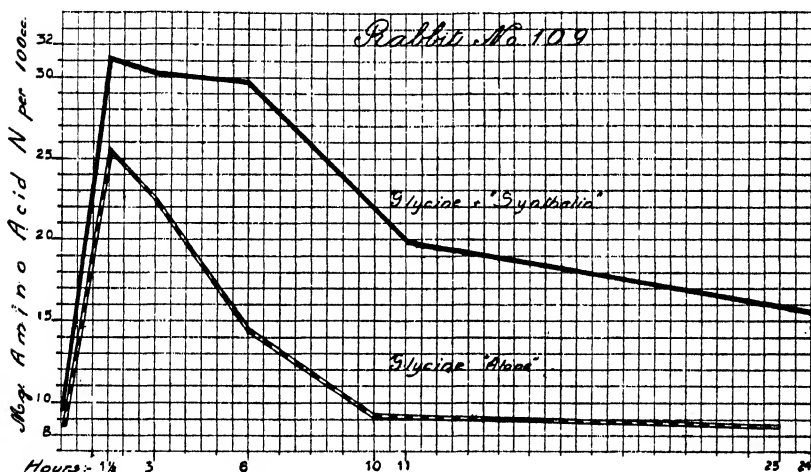
Fig. 1. Decreased ability to deaminize subcutaneously injected glycine when synthalin is administered.

produced an entirely different picture. There was a distinct delay in the return of the amino acids to the normal level. In the cases of Rabbits 109, 118, and A this constituent had not become normal 24 hours after the injections. The urea in every instance showed marked increases due to the accompanying acute nephritis. The blood sugar was decreased in all excepting Rabbit C whose refractoriness to synthalin has been mentioned. Fig. 1 shows the diminished tolerance to glycine produced by synthalin. The values for this one rabbit are characteristic of the others.

The question naturally arises whether this phenomenon truly

represents a diminished ability to deaminize glycine. The complicating factor introduced by the simultaneous injury of the kidneys must be considered. The consensus of opinion appears to be that kidney damage does not ordinarily cause an increase in the amino acids of the blood. This question is discussed by Greene, Sandiford, and Ross (4). These investigators performed a bilateral nephrectomy on a dog and found no increase in the amino acids of the blood during life. Our data support this view. Rabbit 118 was suffering from a spontaneous nephritis as shown by the increased values for blood urea. Nevertheless it responded with a typically normal amino acid curve after the injection of glycine. In order to secure further information on this point, Rabbit U was given an acute nephritis by the injection of uranium nitrate. This animal showed an essentially normal tolerance to injected glycine. We are led to conclude, therefore, that synthalin when injected not only produces an acute nephritis, but also injures the liver and thus interferes with deaminization. An experiment is included in which glycine was administered following the injection of insulin (Rabbit 44). The results show a normal tolerance curve for glycine, a hypoglycemia, and no evidence of renal damage.

Influence of Synthalin on Glucose Tolerance.

Synthalin has a variable influence on the glucose tolerance of rabbits. Rabbit 93 (see Table IV) seems to have utilized or stored all of the glucose administered, as indicated by a continued hypoglycemia. Likewise, Rabbit ·B responded with practically a continuous level of blood sugar after the administration of glucose. On the other hand, Rabbits Y and D experienced hyperglycemia when glucose followed the injection of synthalin. The accumulation of urea in the blood is again evident but the rate of increase is somewhat slower, probably due to the protein-sparing effect of the carbohydrate. The steady increase in the amino acids of the blood following synthalin and glucose in Rabbits 93 and Y is noteworthy. This phenomenon was not observed in the other cases. This striking increase is probably another example of diminished ability to deaminize amino acids as outlined in the preceding section. For comparative purposes an experiment in which insulin and glucose were administered is given (Rabbit 99, Table IV).

TABLE IV.

Glucose Tolerance without and with Synthalin.

Date.	Rabbit No.	Substance.	Initial.	After synthalin.		After glucose.							Remarks.
				1 hr.	2 hrs.	1 hr.	1.5 hrs.	2 hrs.	3 hrs.	6 hrs.	10 hrs.	24 hrs.	
			mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
1927													
May 13	93	Sugar.	87			133		108	87	114	104		*
		Urea N.	26.3	25.7				28.6	28.6	25.7	25.8		
		Amino acid N.	6.8	6.7				6.8	6.8	5.8	7.6		
May 27		Sugar.	95		42		56		74	74	59		† Glucose given 2 hrs. after synthalin. Died during night. Urine from bladder contains much protein.
		Urea N.	24.9		30.3		31.6		37.9	40.5	37.3		
		Amino acid N.	8.5		10.7		12.1		14.7	16.5	17.9		
May 13	Y	Sugar.	80			129		111	118	108	118		*
		Urea N.	33.3			31.7		31.2	33.3	30.3	32.8		
		Amino acid N.	7.8			6.9		6.9	7.1	5.8	7.1		
May 27		Sugar.	95			82	125		133	121	95	65	† Glucose given 1 hr. after synthalin. Died 25 hrs. after injection of synthalin.
		Urea N.	23.8			28.6	29.9		29.6	29.7	38.5	63.3	
		Amino acid N.	7.8			9.1	11.3		9.2	11.9	14.0	18.4	
June 8	B	Sugar.	83			.	105		100	103	98	100	† Glucose and synthalin given at same time.
		Urea N.	29.4				31.7		30.3	34.6	45.0	71.4	
		Amino acid N.	8.7				8.3		7.6	8.8	10.0	9.0	

Date	No.											
June 15	D	Sugar.	100	103		133	125		143	114	108	† Glucose given 1.25 hrs. after synthalin.
		Urea N.	26.6	35.0		38.5	38.5		43.5	68.5	98.0	
		Amino acid N.	9.2	9.1		9.4	8.4		9.6	8.6	7.4	
June 8	99	Sugar.	114			57	52	45	108	100	114	3 units insulin per kilo intravenously and 1.75 gm. glucose per kilo by stomach tube at same time.
		Urea N.	23.5			24.1	24.8	23.7	26.3	24.5	26.6	
		Amino acid N.	8.1			7.5	7.3	7.1	7.1	8.1	8.4	

* Glucose, 1.75 gm. per kilo by stomach tube.

† Synthalin, 4 mg. per kilo intravenously; later, glucose, 1.75 gm. per kilo by stomach tube.

DISCUSSION.

Comparison of our results with those obtained by Lewis with hydrazine reveals some common attributes. This is most striking in the case of the tolerance to injected glycine. Hydrazine and synthalin both interfere with deaminization but hydrazine seems to cause a more severe injury to this mechanism. Hydrazine is sometimes nephrotoxic while synthalin is invariably so after injection. Tolerance for glucose is decreased by hydrazine but it is either increased or unchanged by synthalin.

The hypoglycemia produced by synthalin may be due to a combination of at least two processes. One of these resembles the action of insulin and the other that of hydrazine. The latter substance appears to cause hypoglycemia by injuring the liver and thus preventing normal glyconeogenesis. Synthalin interferes with this mechanism but to a lesser degree. Insulin has no such inhibiting effect. A test which any natural or synthetic material must pass before it is described as insulin-like in action is that it must not interfere with the transformation of non-carbohydrate materials to glucose.

It should be specifically noted that synthalin is prescribed to be taken by the natural route and that our experiments deal with the parenteral administration of the substance. Whether the results which have been described would follow its oral administration we are not in a position to state. Certainly caution should be exercized in using it.

We are indebted to Dr. Fritz E. Bischoff for some of the earlier experiments.

SUMMARY.

Synthalin administered by stomach tube failed to produce hypoglycemia in rabbits. Subcutaneous and intravenous injections usually decreased the blood sugar. An acute nephritis is produced by parenteral administration. There is also an injury of the liver as shown by a decreased ability to deaminize subcutaneously injected glycine. The mechanism of the production of hypoglycemia by synthalin is discussed.

The kidneys and livers of some of the animals which had received doses of 4 mg. of synthalin per kilo were examined microscopically. We are

indebted to Dr. Richard D. Evans for the following report. "In sections of the kidneys stained with hematoxylin and eosin there has been intense damage of the cells lining the convoluted tubules. The injury varies from slight swelling of the cell bodies to complete destruction with granular debris in the lumina. The lumina of some of the convoluted tubules have been obliterated by the intense swelling. There is no noteworthy change of the glomeruli. The livers show a moderate fatty change and the cells about the portal areas are a little shrunken and stain poorly."

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THE PRESERVATION OF BLOOD FOR CHEMICAL ANALYSIS BY THE USE OF SODIUM FLUORIDE.

By JOSEPH H. ROE, OLIVER J. IRISH, AND JAMES I. BOYD.

(From the Department of Chemistry, George Washington University Medical School, Washington.)

Several preservative substances have been recommended for blood that must be kept for a time before an analysis can be performed. Denis and Aldrich (1) proposed the use of 1 drop of formaldehyde to 10 cc. of blood as a preservative for blood sugar. Bock (2) found that five out of six samples of formaldehyde, when used as a blood preservative, gave increased values for blood sugar, the increase being more than 40 per cent in several cases. Ambard (3) advocated the use of a mixture of 2 parts of potassium dihydrogen phosphate and 1 part of sodium fluoride as a blood preservative. Aibara (4) found that 2 mg. of sodium fluoride per cc. of blood had an inhibitory influence upon glycolysis. Sander (5) in a comprehensive investigation of blood preservation found that metallic salts, sodium cyanide, benzoic acid, boric acid, zinc chloride, ether, lysol, phenol, benzene, toluene, acetone, formaldehyde, and thymol, gave unsatisfactory results in maintaining unchanged the values for non-protein nitrogen, urea, uric acid, and sugar, for a period of 48 hours; and that creatinine and creatine remained constant for several days, whether a preservative was used or not. Sander also obtained unreliable results with sodium fluoride in concentrations of 10 mg. per cc. of blood when used alone, but, when combined with thymol, in proportions of 10 mg. of sodium fluoride and 1 mg. of thymol per cc. of blood, he obtained constant values after 10 days standing for sugar, uric acid, creatinine, and creatine, and satisfactory values for urea and non-protein nitrogen, though the latter increased slightly. Denis and Beven (6) used sodium fluoride as a preservative in proportions of 6 mg. per cc. of blood and noted that the concentrations of blood sugar, non-protein nitrogen, urea, creatinine, and uric acid remained unchanged after the samples had been allowed to stand for 48 hours at 28.5–31°C. Major (7) recommended the use of 1 drop of a saturated solution of potassium fluoride to 5 cc. of blood as a preservative. Schwenkter (8) reported satisfactory preservation when a mixture of sodium fluoride and thymol in the proportions recommended by Sander was used as a preservative, but this author obtained values for blood sugar ranging from 7.5 to 50 per cent too high when the Lewis-Benedict picric acid method of analysis was used.

The general conclusion, from the experience of the authors noted above, is that sodium fluoride is an efficient preservative of blood for chemical analysis. Further experimental work seemed desirable, however, upon (1) the concentration of sodium fluoride necessary for satisfactory preservation; (2) the advisability of sterilization when fluorides are used as preservatives; and (3) possible interference with standard methods of analysis. It is the purpose of this paper to report the results of an investigation of these phases of the subject.

All of our experiments were carried out upon human blood. In the preservation tests the bloods were allowed to stand at the

TABLE I.

Amount of Sodium Fluoride Necessary to Prevent Blood Sugar
Decomposition in Sterile Tubes.

No change in blood sugar resulted in 27 days in the tube containing 10 mg. of NaF per cc. of blood.

Mg. sodium fluoride per cc. blood.	Mg. sugar per 100 cc. blood.									
	No. of days standing.									
	0	1	2	3	4	5	6	13	20	27
1	93	90	18	11						
2	90	90	44	35	30					
4	85	85	47	40	24					
6	90	90	93	89	87	89	77	33		
8	103	103	102	108	108	105	108	56	50	
10	95	90	90	96	95	99	93	99	98	98

temperature of the laboratory, which ranged from 22–29°C. The following methods of analysis were used: for sugar, the Rothberg-Evans (9) modification of the Folin-Wu method; for non-protein nitrogen, the method of Wong (10); for urea, the methods of Myers (11), and of Roe and Irish (12); for creatinine, the Folin-Wu (13) method; for uric acid, the Folin (14) method; and for cholesterol, the Bloor (15) procedure.

Our first experiment was to determine the amount of sodium fluoride necessary to prevent decomposition of blood sugar in sterile containers. The results of this experiment are shown in Table I. Samples of blood were placed in sterile tubes containing

1, 2, 4, 6, 8, and 10 mg. of NaF per cc. of blood. Blood sugar values were determined upon these samples, at first daily, later at weekly intervals, for 27 days, except where marked decomposition occurred. The samples containing 1, 2, and 4 mg. per cc. of blood showed unchanged values for 24 hours, but marked glycolysis at the end of the 2nd day; those containing 6 and 8 mg. per cc. showed practically constant values for 6 days but decomposition in 13 days; and the 10 mg. per cc. sample remained unchanged for 27 days, the analysis being discontinued at this point. As there are

TABLE II.

Preservative Influence of Sodium Fluoride in Bloods Kept 10, 20, and 30 Days in Sealed, Sterile Tubes.

Substance determined.	Mg. per 100 cc. blood.															
Sample No.	1		2		3		4		5		6		7		8	
	Containing 10 mg. potassium oxalate per cc.				Containing 10 mg. NaF per cc.											
	Fresh sample.	After 10 days.	Fresh sample.	After 10 days	Fresh sample.	After 10 days.	Fresh sample.	After 10 days.	Fresh sample.	After 20 days.	Fresh sample.	After 20 days.	Fresh sample.	After 30 days.	Fresh sample.	After 30 days.
Non-protein N..	37	40	40	41	34	36	42	42	47	51	27	42	44	57	22	54
Uric acid.......	4.1	4.0	2.4	2.4	3.5	3.2	3.5	3.3	3.4	3.1	3.6	3.3	2.9	2.5	2.9	2.6
Creatinine......	1.3	1.4	1.1	1.2	1.6	1.6	1.7	1.6	1.8	1.8	1.5	1.8	1.7	1.7	1.6	1.6
Sugar...........	116	24	162	20	208	195	150	144	268	254	116	110	217	214	90	97
Cholesterol.....					200	195	222	218	222	211	210	198	175	182	263	250

possibilities of contamination with organisms when removing from a tube samples of blood for analysis, we are unable to say that all of the specimens of blood in this experiment remained completely sterile. The practical conclusion from this experiment is that 10 mg. of NaF per cc. of blood is a satisfactory and desirable concentration to prevent blood sugar decomposition when the blood is collected in a sterile container and efforts are made to prevent the introduction of organisms.

We next made a study of the preservative influence of 10 mg. of NaF per cc. of blood upon other constituents than sugar for pe-

riods of 10, 20, and 30 days under sterile conditions. An experiment was carried out in which a series of duplicate samples of blood was collected in sterile tubes simultaneously from opposite arms of human subjects. One of each of the duplicate samples was analyzed at once and the other was kept sealed until the day of analysis. In this manner bloods were kept sterile for 10, 20, and 30 days, no possibility of contamination existing since the tubes were not opened until the time of analysis. The results are shown in Table II. In the two tubes containing potassium oxalate, definite glycolysis occurred, but the other constituents remained unchanged for 10 days. The values upon the two samples containing

TABLE III.

Blood Sugar Values Obtained with Varying Amounts of Sodium Fluoride in Bloods Inoculated with Bacillus coli communior.

20 mg. of NaF per cc. of blood prevented decomposition for 18 days.

| No. of days standing. | Mg. sugar per 100 cc. blood. | | | | |
| | Mg. NaF per cc. | | | | |
	10	15	20	25	30
0	104	187	152	111	270
3	85	188	146	111	260
7	0	153	152	111	268
11		155	152	113	258
18		80	153		258

NaF, which stood for 10 days before analysis, show satisfactory preservation of non-protein nitrogen, uric acid, creatinine, sugar, and cholesterol, within the limits of experimental error. In the bloods containing NaF, which stood for 20 and 30 days before analysis, the values for sugar, uric acid, creatinine, and cholesterol, remained essentially the same; but the non-protein nitrogen showed a definite increase in one of the 20 day bloods, and in both of the bloods that stood 30 days before analysis. The results of this experiment indicate that satisfactory preservation of sugar, non-protein nitrogen, uric acid, creatinine and cholesterol, may be expected in bloods collected in sterile tubes containing 10 mg. of NaF per cc. of blood for at least 10 days, and

that in bloods submitted to the same conditions preservation of sugar, uric acid, creatinine, and cholesterol, but not non-protein nitrogen, will occur for 30 days.

Having determined the amount of sodium fluoride necessary to give successful preservation of bloods kept in sterile containers, we next made a study of the influence of sodium fluoride under non-sterile conditions. An experiment was performed in which 10, 15, 20, 25, and 30 mg. of NaF per cc. of blood were used and each specimen was inoculated with a loopful of a pure culture of *Bacillus coli communior*. The results of this experiment are shown in Table III. Some decomposition occurred by the 3rd day in the

TABLE IV.

Influence of Sodium Fluoride upon Two Samples of Blood Containing 20 Mg. of NaF per Cc. When Inoculated with Bacillus coli communior.

No. of days standing.	Mg. per 100 cc. blood.							
	Sugar.		Uric acid.		Non-protein N.		Creatinine.	
	Sample 1.	Sample 2.	Sample 1.	Sample 2.	Sample 1.	Sample 2.	Sample 1.	Sample 2.
0	142	129	3.73	3.67	36	37	1.10	1.10
4	143	129	3.42	3.40	36	47	1.16	1.30
7	145	127	3.27	3.26	47	51	1.26	1.44
10	141	125	3.57	3.63	44	54	1.40	1.61
17	141	126	3.57	3.49	62	63	1.41	1.62
24	137	123	3.63	3.55	66	76	1.83	1.81

tube containing 10 mg. of NaF per cc. of blood and the sugar disappeared completely in this specimen by the 7th day. With 15 mg. of NaF per cc. the blood sugar value remained unchanged for 3 days, but decreased by the 7th day, and by the 18th day was less than half the initial value. The values remained practically the same in the samples containing 20, 25, and 30 mg. of NaF per cc. of blood. Similar results were obtained in an experiment in which samples of blood containing 10, 15, and 20 mg. of NaF per cc. were inoculated with *Bacillus subtilis*. In the presence of this organism the specimen containing 20 mg. of NaF per cc. showed unchanged blood sugar values for 28 days; and the samples

containing 10 and 15 mg. per cc. showed undesirable decomposition in less than 7 days. These data demonstrate that 10 mg. of NaF per cc. of blood are inadequate to prevent decomposition of blood sugar in the presence of two common contaminating organisms, *Bacillus coli communior* and *Bacillus subtilis*; and that at least 20 mg. of NaF per cc. of blood are necessary to maintain unchanged values for blood sugar in non-sterile blood.

In Table IV is shown the influence of sodium fluoride upon the non-protein nitrogen, uric acid, creatinine, and sugar, of bloods inoculated with *Bacillus coli communior*. As 20 mg. of NaF per cc. of blood had prevented decomposition of sugar in non-sterile bloods (Table III), this concentration was used to determine whether preservation of other constituents than sugar would result under non-sterile conditions. Two large pooled samples containing 20 mg. of NaF per cc. were inoculated with a pure culture of colon bacilli. Initial values for sugar, uric acid, non-protein nitrogen, and creatinine, were obtained, and the samples were then analyzed upon the 4th, 7th, 10th, 17th, and 24th days following collection. Table IV shows that sugar and uric acid were preserved satisfactorily for 24 days and that the non-protein nitrogen and the reducing substance determined as creatinine gradually increased. A similar increase in non-protein nitrogen was obtained in bloods inoculated with *Bacillus subtilis*. This increase in non-protein nitrogen was expected since a similar, though much less rapid, change had been noted in this constituent when the blood was kept under sterile conditions (Table II). Denis and Beven (6) also noted an increase in non-protein nitrogen in bloods treated with 6 mg. of NaF per cc. of blood after 96 hours standing. The increase in the value for creatinine is unusual and has not been noted by us in any other experiments than those in which the bloods were inoculated with colon bacilli. This experiment shows that 20 mg. of NaF per cc. of blood will prevent the decomposition of sugar and uric acid in bloods inoculated with *Bacillus coli communior* and that non-protein nitrogen and the substance determined as creatinine will gradually increase in bloods submitted to the same conditions.

The results we have obtained with sodium fluoride as a blood preservative are not in agreement with the work of Sander (5) in several respects. This author found that neither sodium fluor-

ide nor thymol, when used alone, gave satisfactory preservation, but when these two substances were combined and used in a concentration of 10 mg. of NaF and 1 mg. of thymol per cc. of blood, successful preservation resulted even in non-sterile containers. In our experiments, 10 mg. of NaF per cc. of blood gave very satisfactory preservation for periods of 10 days, or more, in bloods collected in sterile containers; but in non-sterile bloods neither 10 mg. of NaF, nor 10 mg. of NaF and 1 mg. of thymol, per cc. of blood, resulted in the preservation of blood sugar. The failure of a mixture of NaF and thymol to give preservation of sugar in non-sterile bloods was demonstrated in an experiment in which three samples of blood containing 10 mg. of NaF and 1 mg. of thymol per cc. were inoculated with *Bacillus coli communior*. Blood sugar estimations were carried out upon these bloods at once and upon the 3rd, 7th, and 11th days thereafter. Definite decomposition was shown in the 3rd and 7th day analyses and upon the 11th day the sugar had practically disappeared. These data indicate that thymol contributes little additional preservative power to sodium fluoride when combined with the latter. Since thymol interferes with one of the standard methods of analysis for sugar (Schwenkter), its use as an adjunct to sodium fluoride seems contraindicated.

Sander (5) also states that: "It is evidently not necessary to draw blood under sterile conditions, except with regard to the patient, in order to preserve blood samples with fluoride and thymol." Our experiments indicate that sterilization is very important in obtaining successful preservation of blood. With 10 mg. of NaF per cc. of blood we obtained entirely satisfactory preservation of sugar, non-protein nitrogen, uric acid, creatinine, and cholesterol, for at least 10 days, in sterile bloods; but only partially successful preservation was secured in non-sterile bloods when even twice as much NaF was used. We would emphasize that sterile conditions, while possibly not necessary to secure a certain degree of preservation, are certainly very desirable in connection with the use of sodium fluoride as a blood preservative.

We have found no interference by sodium fluoride in several times the concentration necessary as a preservative in the methods of analysis used, except in the determination of urea by urease hydrolysis. Sodium fluoride definitely retards the hydrolysis of

urea to ammonium carbonate by urease action. This is not an unexpected finding since fluorides inhibit glycolysis and probably manifest other antienzymic powers in their action as preservatives.

The inhibition of urease action by sodium fluoride is best demonstrated by adding the latter to a pure urea solution and determining the urea by a urease method. With such experiments we found that NaF interferes in the determination of urea by urease hydrolysis in a degree proportional to the concentration of

TABLE V.

Influence of Sodium Fluoride upon Urease Action.

A pure urea solution containing 30 mg. of urea nitrogen per 100 cc., buffered with 0.25 M K_2HPO_4 and KH_2PO_4, was used. Each tube was treated with the same amount of the same urease solution and incubated 1 hour at 40°C.

Mg. sodium fluoride per cc. hydrolysate.	Mg. urea nitrogen per 100 cc. solution.	Per cent loss.
0	29.6	0
0	29.6	0
0	30.3	0
0.5	29.8	0
1.0	27.8	7.3
1.5	22.4	25.4
2.0	15.5	48.4
2.5	10.5	65.0
3.0	8.2	72.7
3.5	6.1	79.6
4.0	5.6	81.3
4.5	3.8	87.3
5.0	3.0	90.0
7.5	Not determinable.	
10.0	" "	

fluoride. This is shown in Table V. In this experiment a pure urea solution containing 30 mg. of urea nitrogen per 100 cc., buffered with 0.25 M KH_2PO_4 and K_2HPO_4, was used. Aliquot parts of this solution were placed in each of a series of tubes and water and NaF were added to each tube in amounts to make concentrations of 0.5 to 10 mg. per cc. of solution. The urea in each of these solutions was then determined by urease hydrolysis, the conditions of the hydrolysis being made identical in each

determination. The recoveries of urea obtained were in an inverse proportion to the concentration of NaF, the losses ranging from 7.3 per cent, where 1 mg. of NaF per cc. of hydrolysate was present, to about 99 per cent, where the concentration of NaF was 10 mg. per cc. of hydrolysate.

In attempting to overcome this objection to the use of NaF as a preservative, we found two procedures to be beneficial: (1) dilution of the blood; (2) precipitation of the fluoride ions with magnesium.

Since the speed of urease hydrolysis in the presence of NaF is retarded in a degree proportional to the concentration of fluoride, it follows that dilution should favor completion of the hydrolysis, and thus bring about more complete recoveries of urea nitrogen in bloods preserved with this substance. This was demonstrated to be true in an experiment in which NaF was added to samples of the same blood in concentrations of 10, 20, 30, 50, 75, and 100 mg. per cc. of blood. These bloods were then diluted with 7 volumes of distilled water and their urea nitrogen was determined by urease hydrolysis. The samples containing 10, 20, and 30 mg. of NaF per cc. of blood gave the same value for urea as control samples containing oxalate only. Low recoveries were obtained with the samples containing concentrations of NaF higher than 30 mg. of NaF per cc. in this and other experiments. The inhibitory effect in this experiment was less marked than that noted in experiments with water solutions of urea. A concentration of 1 mg. of NaF per cc. of hydrolysate gave recoveries 7.3 per cent too low in the experiment with aqueous urea solution; but in the experiment with blood a concentration of 3.75 mg. of NaF per cc. of diluted blood was tolerated without interference. Less interference in the case of blood is due to the precipitation of fluoride ions by calcium and magnesium, and probably to chemical combination with other constituents of the blood.

Dilution with water is obviously limited in its possibilities, because above a certain limit increasing the volume of water makes the procedure impractical. However, for concentrations of NaF up to 30 mg. per cc. of blood dilution with 7 to 10 volumes of water is all the treatment that is necessary to secure complete hydrolysis of urea. Above this concentration complete recoveries may be obtained by the addition of magnesium sulfate solution, the

amount of magnesium added being in slight excess of that necessary to precipitate the fluoride ions. Of the fluoride precipitants examined magnesium was found to be the most satisfactory. Calcium will precipitate fluoride ions, but cannot be used, because it precipitates the phosphate used as a buffer. Magnesium does not precipitate phosphate ions at the pH of the blood and, when added in modrate excess, does precipitate fluoride ions. Magnesium may thus be used to precipitate fluoride ions and remove the interfering action of the latter, but is not necessary unless concentrations greater than 30 mg. of NaF per cc. of blood are used. Below this concentration of fluoride dilution with 7 to 10 volumes of water before treatment with urease is adequate to secure complete recoveries of blood urea by the urease methods of analysis.

SUMMARY.

1. It has been found that sodium fluoride in concentrations of 10 mg. per cc. of blood will prevent changes in the values for nonprotein nitrogen, uric acid, creatinine, sugar, and cholesterol, in sterile blood for at least 10 days.

2. A concentration of at least 20 mg. of NaF per cc. of blood has been found necessary to prevent marked changes in non-sterile blood, but even increased amounts of fluoride do not give as satisfactory preservation as can be obtained with blood submitted to sterile conditions.

3. Sodium fluoride interferes with the determination of urea by methods involving urease hydrolysis. The interference is proportional to the concentration of fluoride in the hydrolysate. This difficulty can be overcome in bloods containing not over 30 mg. of NaF per cc. by diluting with 7 to 10 volumes of water previous to treatment with urease.

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THE DISTRIBUTION OF ELECTROLYTES IN INTESTINAL OBSTRUCTION.

By DANA W. ATCHLEY AND ETHEL M. BENEDICT.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

In spite of the great volume of work in this field, the only complete chemical study of the disturbances in intestinal or pyloric obstruction is that made by Gamble and Ross (1) in 1925. These authors presented the first integrated picture of the mechanism as a whole and offered convincing explanation of the accompanying phenomena on the basis of simple physiological laws. We have been able to make somewhat more complete partitions of the serum electrolytes in two experimental animals and one clinical case of intestinal obstruction, and desire to offer our evidence to confirm their results. Among other observations, Gamble and Ross found a striking increase in the undetermined acids (R) of the plasma, an increase which they felt must be, in part, due to an increase in organic acids. We have been able to explain this entire change by means of the inorganic anions, phosphate and sulfate.

EXPERIMENTAL.

The chemical methods, the procedure for obtaining and preparing the serum, and the calculations have been described in a previous paper (2). There is one exception; base bound to protein was derived by a formula determined by Hastings *et al.* (3) for human blood, 2.03 P^1 at pH 7.35.

Dog 25 was a male weighing 14.3 kilos; the jejunum was ligated with tape about 45 cm. from the pylorus (ether anesthesia). The animal was quite sick for 3 or 4 days but made a spontaneous recovery. Blood was taken on the 4th day at the height of the

[1] P = protein per cent.

TABLE I.

		Serum.									Whole blood.			
Date.	Non-protein N.	Protein.	Total base.	Total acid.	B-A	Chloride.	Carbonate.	Phosphate.	Protein.	Sulfate.	Hb.	Hemato-crit.	Weight.	
1927	*mg. per 100 cc.*	*per cent*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*m.-eq. per l.*	*per cent*	*vol per cent*	*kg.*	
Dog 25.														
Apr. 18	25	5.6	157.8	151.4	6.4	115.0	22.8	2.2	11.4		76	32.0	14.3	Total loss of fluids in vomitus = 2380 cc. Total loss of base in vomitus = 181.5 m.-eq. Total loss of chlorides in vomitus = 158.5 m.-eq.
" 21	100	8.0	160.0	152.6	7.4	102.8	27.2	2.9	16.4	3.3	99	42.0	12.1	
Dog 26.														
Apr. 26	18	7.1	152.2	147.3	4.9	108.4	22.9	1.6	14.4		62	26.3	9.3	Total loss of fluids in vomitus and urine = 1730 cc. Total loss of base in vomitus, etc. = 120.7 m.-eq. Total loss of chlorides in vomitus, etc. = 135.6 m.-eq.
" 29	172	9.7	136.8	130.7	6.1	61.0	40.1	3.9	19.7	6.0	71	29.2	8.0	
E. S., Wd.8, 68289.														
Mar. 18	150	9.6	135.0	123.9	11.0	68.2	26.6	3.6	19.5	6.1	115	43.4		
" 19	125	7.3	137.9	134.1	3.8	83.4	28.1	2.7	14.8	5.1	86	35.0		
" 22	31	6.9	139.9	135.5	4.4	93.8	26.8	0.9	14.0		86	33.2		
" 29	18	6.7	145.2	142.8	2.4	100.8	26.6	1.8	13.6		74	31.2		

illness. The data on this and the other experiments will be found in Table I.

In Dog 26, a female weighing 9.3 kilos, the intestine was severed 10 cm. below the pylorus and both ends were inverted. The animal recovered well from the operation but became very ill and an autopsy was performed the 4th day. The stomach and intestine above the obstruction contained about 125 cc. of fluid with 1.8 m.-eq. of base and 1.5 m.-eq. of chloride. The combined vomitus and urine were analyzed for base and chloride content (Table I).

The patient whose blood analyses appear in Table I may be described briefly as follows:

Presbyterian Hospital History 68289, a woman, aged 58 years, admitted March 17, 1927; operated upon the same day and discharged April 8, 1927. The previous history is irrelevant. 5 days before admission she was suddenly seized with cramp-like pains around the umbilicus and soon began to vomit; these symptoms became increasingly severe, continuing to her admission. She had no bowel movements during this 5 day period.

Physical examination showed her acutely ill, but there were no significant abnormalities outside the abdomen which was distended, tense, immovable with respiration, and dull in the flanks. White blood cells, 27,000. Immediate exploration revealed a strangulated intestine due to a Richter type of direct inguinal hernia. This was reduced and an ileostomy performed.

Normal saline solution was administered and improvement began at once. Recovery was relatively uneventful. Four blood analyses were made. All the results are given in Table I.

DISCUSSION.

Our results confirm the well known blood changes in this condition: (1) dehydration, (2) nitrogen retention, (3) loss of chloride, and (4) increase in carbonate. We find, also, the decrease in serum base reported by Gamble and Ross (1), as well as the relatively (to chloride) high loss of base in the vomitus (Dog 25). But it is possible to go somewhat further in an analysis of the various changes that occur. First of all, the increase in phosphate, sulfate, and protein anions easily accounts for the large increase in undetermined acid (R) found by Gamble and Ross (1). For example, in Dog 26, the increase in phosphate was 2.3 m.-eq., in sulfate about 5.5 m.-eq. (assuming a normal of 0.5 m.-eq.), and in

protein 5.3 m.-eq.   This makes a total change of 13.1 m.-eq.
Dog 25 was a milder case but even here these three anions to-
gether caused a rise of 8.5 m.-eq.   In both dogs there was no signifi-
cant change in undetermined acid (R of Gamble and Ross; B − A
in Table I) after obstruction, a fact which means that one need
not assume any change in organic acid concentration.   In the
human case there is a real difference (7.2 m.-eq.) between the
B − A of the first and second observations.   We are unable to
explain this change.   It is possible that the reaction of the blood
was quite different on the two occasions and there may have been
fairly large quantities of ketones present on the 1st day.   However,
these points are not clarified by our data.

It is rather interesting to analyze the various processes that
contribute to the striking decrease in serum chlorides.   The chlo-
ride concentration in Dog 26 fell from 108.4 m.-eq. to 61.0 m.-eq.,
a drop of 47.4 m.-eq.   Of that total, 15.4 m.-eq. represent loss of
BCl, for the total base decreased that amount, a loss which is
probably due, in main, to vomiting of BCl (although a little BCl
must have been excreted in the urine).   The increase in carbonate
of 17.2 m.-eq. suggests that a corresponding amount of base was
freed by the excretion of HCl into the stomach with subsequent
vomiting.   Carbonate was used to cover this base to the extent of
17.2 m.-eq.   Altogether, therefore, removal of chlorides from the
body clearly and directly accounts for 32.6 m.-eq. of the total loss
of 47.4 m.-eq.   The remaining 14.8 m.-eq. is more complicated.
There is an increase in protein concentration of 5.3 m.-eq. due to
dehydration, and there is also a striking retention of phosphate and
sulfate, caused by renal insufficiency.   The combined increases
due to these two anions is 7.8 m.-eq.   There are then 13.1 m.-eq.
dependent directly or indirectly on dehydration (assuming the
renal insufficiency to be due to dehydration).   The small amount
of chloride unaccounted for is well within the errors of the methods.
The effect of sulfate and phosphate retention on serum chlorides
has been pointed out by the authors in a recent paper (2).   It was
found that in the acidosis of acute renal insufficiency (ligated
ureters in dogs) chloride plays a more significant protective rôle
than carbonate, rendering twice as much base to the retained
acids as does carbonate.   The mechanism of this chloride neutral-
ization is difficult to explain but apparently is independent of

chloride loss from the body. Moreover, tissue analyses of muscle, brain, and skin showed a drop in chloride paralleling that of the blood, as White and Bridge (4) and Haden and Orr (5) have shown for other tissues in obstructed animals. It is not a redistribution of chloride from blood to tissues. Studies of Dog 25 and the human case show pictures similar to that described above. It is possible to say, therefore, that the major factors governing the disappearance of serum chlorides in intestinal obstruction are (1) loss of BCl and HCl in vomitus (and urine) and (2) replacement of chloride. by increase in the protein, sulfate, and phosphate anions—due essentially to dehydration. Moreover, it seems unlikely that there is place in such a mechanism for the neutralization by chloride of a hypothetical toxin, as is suggested by Haden and Orr (6).

While we were primarily interested in the chemical aspects of intestinal obstruction, one clinical feature seemed interesting enough to deserve comment. The limited nature of our data is apparent and the suggestion requires much confirmation. However, the most consistent and striking change between the first and second bloods on our patient was the great dilution of the serum as indicated by protein per cent, hematocrit, and hemoglobin. This chemical change was coincident with a tremendous clinical improvement and one is led to infer that a simple determination of serum protein per cent might be the best aid in following treatment. 4 days after the first study, the patient's total base was still very low and the chlorides far below normal yet she was practically well. In other words, may the clinical course of intestinal obstruction as far as its general effects are concerned, not parallel more closely the water content of the serum than the distribution of electrolytes or even the retention of nitrogen? Further observations are being made in order to arrive at an answer to this question.

<div align="center">SUMMARY.</div>

Electrolyte partitions of the sera of one patient and two dogs with intestinal obstruction are reported.

The essential changes during obstruction were (1) decrease in total base, (2) decrease in chlorides, (3) increase in carbonates, protein anions, sulfates, and phosphates, (4) dehydration as determined by serum protein per cent, hematocrit, and hemoglo-

bin. There is no consistent change in undertermined acids $(B - A)$ and therefore no necessity for assuming changes in organic acid content.

Analysis of the factors causing the disappearance of chlorides indicates that this change is due (1) to loss of BCl and HCl by vomitus, and (2) to replacement of the base bound to chlorides by acids (phosphate and sulfate) retained because of renal insufficiency. Results in experimental renal insufficiency reported elsewhere make it doubtful that this latter change is brought about by actual loss of chlorides from the body.

It is tentatively suggested that the beneficial results of non-operative treatment on the clinical course of intestinal obstruction are best followed by their effect on the dehydration of the blood.

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ON THE PREPARATION AND PROPERTIES OF PURE GLUTATHIONE (GLUTAMINYL-CYSTEINE).

BY J. M. JOHNSON AND CARL VOEGTLIN.

(*From the Division of Pharmacology, Hygienic Laboratory, United States Public Health Service, Washington.*)

Glutathione was first isolated in 1921 from yeast, mammalian liver, and muscle by Hopkins (5), who also presented evidence indicating that the substance is a dipeptide of cysteine and glutamic acid. In 1925 Stewart and Tunnicliffe (8) published their work on the synthesis of glutathione. Their second synthetic method yielded a product which possessed all of the properties of the substance found in nature. Since then, a number of other workers have prepared the natural product, but so far no analytic findings have been published which would support the data given by the English investigators. Brand and Sandberg (3), for instance, obtained a product from ox blood with a sulfur content of 8.6 per cent instead of the theoretical figure of 12.87 per cent. They therefore considered their substance impure. The other workers, with the exception of Hunter and Eagles (7), do not give any analytical findings at all. About a year ago there appeared a paper by Hunter and Eagles (7) which led these workers to the conclusion that glutathione prepared from yeast, blood, and liver in reality is not a simple dipeptide composed of cysteine and glutamic acid, but more likely a tripeptide of unknown composition. They base this conclusion largely on the fact that their products had a much lower sulfur and higher total nitrogen content than the products of the English workers. Hunter and Eagles' report was immediately followed by a short note by Hopkins (6) in which he suggests that the low sulfur content of the products obtained by Hunter and Eagles was probably due to the splitting off of sulfur from the substance by the alkali used in the course of isolation. Bergmann and Stather (2) had previously called attention to the great ease with which alkali splits off sulfur from dialanyl-*l*-cystine dianhydride. Referring to this work Hopkins in his note reported some experiments which indicate that exposure of pure glutathione to alkali results in the splitting off of sulfur. He concludes with the following remarks: "In any case, although I have myself no doubt as to the nature of glutathione, the appearance of Hunter and Eagles' papers makes it desirable that I should if possible give greater precision to the account of its isolation. This I hope to do in the near future."

During the last 5 years we have had a large amount of experience in the isolation of glutathione, which material we used in work dealing with the physiological and pharmacological action of the substance. Suffice it to say here that we worked up twenty-three batches of pressed yeast, totaling over 1000 kilos. The purpose of the present communication is to report briefly our findings in view of the fact that a report on the subject by independent investigators is needed to clear up the discrepancy between the published claims. We may state at once that we have never had any reason to doubt the reliability of the statements of the English workers as to the structure of glutathione. On the other hand we may state that in our earlier experience we sometimes obtained products which analyzed low in sulfur and high in total nitrogen. These products were obtained by following Hopkins' method in every essential detail to the best of our ability. Hence it was obvious that the method described by Hopkins may or may not yield a pure product and our efforts were therefore turned towards modifying the method in such a way as to avoid the production of impure material. In the experimental part we shall presently describe this method as accurately as possible so that other workers may have no difficulty in repeating the work. It is well here to emphasize the fact that the isolation can only be satisfactorily accomplished with patience and careful observation of every required detail. It will be seen that our description of the method deviates only slightly from that given by Hopkins, but we are convinced that it will overcome the difficulties inherent in his method. In addition we report the preparation and analysis of some compounds of glutathione with certain heavy metal salts. $S-S$ glutathione always indicates the oxidized form and SH glutathione the reduced form.

<div align="center">EXPERIMENTAL.</div>

Preparation of Glutathione from Yeast.—Fresh, pressed bakers' yeast (45 kilos), free from starch,[1] is dropped in small pieces into 100 liters of water previously heated to about 80°C. The mixture is brought to the boiling point under constant stirring, cooled, and

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[1] Ordinary commercial bakers' yeast contains a considerable amount of starch, which in our experience renders the first extraction very difficult.

the extract separated by means of a Sharples centrifuge. The solid residue is again extracted with 50 liters of boiling water. The combined extracts are cooled to about 15°C. and a cold saturated solution of neutral lead acetate is added to complete precipitation. Concentrated NH_4OH is added until the mixture is only *slightly acid* to litmus. After standing overnight at ordinary temperature, the lead precipitate is separated by means of the Sharples centrifuge and washed with a small volume of cold water. The precipitate is ground up in mortars with the addition of pure quartz sand and 0.5 N H_2SO_4 until the filtrate contains a slight excess of free H_2SO_4. The $PbSO_4$ is filtered off on Buchner filters. Further extraction of the residue with H_2SO_4 does not increase the yield in glutathione, though such extract still gives a nitroprusside test and material precipitable with $HgSO_4$. The acid extract, amounting to about 10 to 12 liters, is treated in 2 liter portions with a saturated solution of uranium acetate until a little of the filtered fluid gives a strong color with potassium ferrocyanide. The mixture after thorough cooling with an ice-salt mixture is treated with a hot saturated solution of $Ba(OH)_2$ and filtered *without delay and as rapidly as possible* on several Buchner filters. The filtrate is immediately acidified with a slight excess of H_2SO_4 and filtered. The filtrate is now precipitated with the Hopkins-Cole $HgSO_4$ reagent, avoiding an excess of the reagent. After standing for several hours the precipitate is collected on a Buchner filter, well washed with cold water, suspended in water, and decomposed with H_2S. The filtrate (1200 cc.) from the HgS is aerated to remove H_2S, made roughly 0.5 N with H_2SO_4, and precipitated with an excess of a saturated solution of phosphotungstic acid in 0.5 N H_2SO_4. A small amount of gummy precipitate is filtered off and the phosphotungstic acid removed from the cooled filtrate by the addition of a *cooled, saturated* solution of $Ba(OH)_2$. The mixture is filtered *as rapidly as possible* on a Buchner funnel and the filtrate acidified at once with H_2SO_4 and again precipitated with $HgSO_4$ in the manner previously described. After standing for several hours the mercury precipitate is collected on a Buchner filter, washed several times with H_2O, and finally suspended in about 100 cc. of H_2O. After complete decomposition with H_2S and removal of HgS by filtration and washing of the latter with water, the combined filtrates are aerated with purified (NH_3-free) air in

order to remove H₂S. The solution is then carefully adjusted so as to be free from both H₂SO₄ and barium and is concentrated under reduced pressure (below 50°C.) to a thin syrup. This syrup and watery washings from the distilling flask, amounting to 10 cc., are poured into a cooled mixture of 100 cc. of absolute alcohol and 100 cc. of dry ether. After standing for about 12 hours in the ice box the supernatant alcohol-ether mixture is poured off and the somewhat gummy precipitate treated with 100 cc. of absolute alcohol. After standing for another 12 hours the precipitate is friable and is removed to a vacuum desiccator and dried over P₂O₅ and paraffin to constant weight. Yield of SH glutathione, 4 gm.

Conversion of SH Glutathione into S−S Glutathione.—500 mg. of the above SH glutathione are dissolved in 15 cc. of H₂O and carefully made *very slightly alkaline* to litmus by the addition of cold saturated Ba(OH)₂ solution. A rapid stream of pure oxygen is run through this solution until the nitroprusside test is just negative (in this case after 2 hours). *Without delay* the solution is freed from barium with H₂SO₄, an excess of the latter being avoided. The use of the centrifuge for testing the freedom of the solution from both barium and H₂SO₄ is time-saving. After concentration to a thin syrup (3 cc.) under reduced pressure (below 50°C.) the material is precipitated by being poured into 40 cc. of absolute alcohol. The precipitate becomes friable after standing overnight. It is dried in a vacuum desiccator over P₂O₅ and paraffin and finally brought to constant weight at 100°C. *in vacuo* over P₂O₅. Yield of S−S glutathione, 450 mg.

Analytical Methods.—Total nitrogen was estimated by the Kjeldahl method; the total sulfur by the method described by Hoffman and Gortner (4), use being made of an electric furnace for the ashing. This method was first tested as to its reliability by applying it to the analysis of c.p. cystine and cysteine hydrochloride with satisfactory results. The specific rotation was determined by means of a Schmidt and Haensch polarimeter, either mercury light or an electrical bulb (Mazda) of 500 watts being used.

The gold was estimated as the metal after ashing of the compound in the electric furnace at 500°C. The copper was estimated as CuO after fusion in an electric furnace at 500°C. with a mixture of NaNO₃ and NH₄NO₃, followed by precipitation with KOH.

The lead was determined as $PbSO_4$, by treating the compound with dilute H_2SO_4, concentration on the water bath, and heating over the free flame until fumes of H_2SO_4 came off. After cooling and dilution with water the precipitate was collected on a Gooch filter.

Copper, Gold, and Lead Derivatives of SH Glutathione.—To 500 mg. of SH glutathione, dissolved in 20 cc. of H_2O, there are added 5 cc. of a solution of $CuCl_2$ containing 169.3 mg. of $CuCl_2$ + $2H_2O$, *i.e.* 4 mols of glutathione to 2 mols of $CuCl_2$. The gradual addition of 6.5 cc. of 0.25 N NaOH produces a grayish green precipitate which is separated by centrifugation and is washed free from chlorides by water. The addition of more 0.25 N NaOH to the mother liquor does not cause any further precipitation. The precipitate is dried to constant weight over P_2O_5 and represents an amorphous grayish green powder. Yield, 82 mg.

Found. Cu 33.20 per cent. Calculated for $C_8H_{12}O_5N_3SCu_2$. 33.88 per cent.
"   S   8.07 "   "   "   "   "   8.55 "   "

To 500 mg. of SH glutathione, dissolved in 7.5 cc. of H_2O, there is gradually added a solution of $AuCl_3$, previously neutralized with NaOH, until no further precipitation occurs. This requires $\frac{1}{3}$ of an atom of Au to 1 mol of glutathione. A very small amount of precipitate was centrifuged off and discarded. The pale brown supernatant fluid is precipitated by the addition of 10 cc. of absolute alcohol. The precipitate is separated by centrifugation, washed once with absolute alcohol, and dried to constant weight over P_2O_5 and paraffin. Yield, 258 mg. of a white, amorphous powder, which is easily soluble in water.

Found. Au 43.75 per cent. Calculated for $C_8H_{13}O_5N_3SAu$. 44.20 per cent.
"   S   6.52 "   "   "   "   "   7.21 "   "

From the mother liquor of the above gold compound there are recovered (after removal of the gold) by mercury precipitation 220 mg. of glutathione.

To 200 mg. of SH glutathione, dissolved in 5 cc. of H_2O, there is added gradually a solution of basic lead acetate (Goulard's extract) until complete precipitation has occurred, which requires 1.25 cc. The precipitate is separated by centrifugation, washed

repeatedly with water, and dried to constant weight in a vacuum desiccator over P_2O_5.   Yield, 276 mg. of a white amorphous powder.

Found.   Pb 44.92 per cent.   Calculated for $C_8H_{12}O_5N_2SPb$.   45.50 per cent.
    "   N   6.96 "   "   "   "   "   6.15 "   "

DISCUSSION.

Preparation.—In our experience fresh, pressed, and starch-free bakers' yeast is the most suitable raw material for the preparation of pure glutathione, though we have found brewers' yeast (bottom) quite satisfactory. Yeast is superior to ox liver, as the preliminary extraction is rather cumbersome with ox liver. We found a single extraction with water quite sufficient in removing all but a small amount of the substance. The use of a Sharples centrifuge in the preliminary extraction and for the separation of the lead precipitate is very convenient and time-saving and it is quite feasible to obtain the lead precipitate from 45 kilos of yeast ready for acid decomposition within 12 to 36 hours. *Particular care is required to speed up the steps in the process when the material is in contact with alkali, and the temperature should be lowered during these stages by proper cooling.* Contrary to Hunter and Eagles, and confirming Hopkins, we consider the precipitation with phosphotungstic acid essential for the removal of impurities. We have eliminated the precipitation with copper or lead following the second mercury precipitation, as in our experience these reagents do not yield a pure product if at this stage the impurities have not been removed. We have repeatedly attempted to purify products low in sulfur by precipitation with copper, mercuric sulfate, or lead acetate without success, as analysis of the final products showed. Once formed, these impurities cannot be removed by the present methods. We also found that it is easier to obtain SH glutathione in pure form than S—S glutathione. We are inclined to attribute this to the possibility of sulfur being split off during the final oxidation. In fact we have shown that a given sample of pure SH glutathione will yield S—S glutathione low in sulfur if the solution is not worked up *as soon as the nitroprusside test has disappeared.* A few hours standing in even a slightly alkaline medium will lead to deterioration. We also recommend using

relatively small batches (0.5 to 1 gm.) of SH glutathione for carrying out the oxidation.

Yield.—The yield of pure substance we found to be about the same as that given by Hopkins (100 to 150 mg. per kilo). This represents only a fraction of the substance present in pressed yeast (containing about 25 per cent solids) as indicated by iodine titration of a trichloroacetic acid extract. The low yield is partly explained by the incomplete precipitation of the substance by lead acetate, copper salts, and mercuric sulfate, which is evident from a consideration of the experiments described by us, where every precaution was taken to obtain complete precipitation. In other preparations of these metal derivatives somewhat different yields were obtained, but in every case the loss of substance was considerable. We believe that this loss in the copper precipitation is partly due to the conversion of part of the SH glutathione into $S-S$ glutathione by the copper salt, and $S-S$ glutathione is not precipitated by copper. A similar, though perhaps smaller loss occurs in the use of lead and mercury, due undoubtedly to the solubility of these metal derivatives. At any rate we have often recovered from the filtrates of the heavy metal precipitates, after removal of the metal with H_2S, considerable amounts of glutathione. The alcohol-ether mother liquors in the final precipitation also yield some additional substance after evaporation of the solvent, but as a rule this is only a small amount and of uncertain purity.

Purity.—The results in Table I are given as an illustration of the agreement in the analytical findings of two of our products with those of Hopkins, and Stewart and Tunnicliffe. As stated by Hopkins, the behavior of the substance in the melting point determination is of minor significance as glutathione has no sharp melting point. Making allowance for this fact it will be seen that our figures agree with those given by Hopkins for $S-S$ glutathione.

The figures for total nitrogen and sulfur are in perfect agreement with those of Hopkins and those required by theory. Our sulfur values have always been *slightly* lower than the theoretical, but this is to be expected.

The figures for the optical rotation of $S-S$ glutathione are somewhat lower than those given by Stewart and Tunnicliffe for the

natural and synthetic product; *i.e.*, $-93.9°$ in aqueous solution and $-84.7°$ in 10 per cent HCl instead of $-98.3°$ and $-89.2°$ respectively. The difference of the rotation in water and in acid is in both cases the same, 9.1 to 9.2. We used for these determinations the same concentrations and the same light as the

TABLE I.

Physical Properties and Analytical Findings from Yeast Glutathione.

		S—S glutathione.		SH glutathione.	Calculated.
		Hopkins.	Johnson and Voegtlin.	Johnson and Voegtlin.	
Melting point. { Softening at... Swelling at....		165–167°C. 182–185 "	162–163°C. 170–195 "	111°C. 185–190°C. (melts).	
Total nitrogen, *per cent*		11.70	11.24	11.14	11.24
Sulfur, *per cent*.................		12.31	12.06	12.33	12.85
		Stewart and Tunnicliffe.	Johnson and Voegtlin.	Johnson and Voegtlin.	
Specific rotation. $[\alpha]_{Hgj}$ 3.46 per cent in H_2O at 28.5°C.			$-93.9°$		
1.73 " " " 10 per cent HCl at 26.6°C.....................			$-84.7°$		
Electric light. 3.46 per cent in H_2O at 28.5°C.			$-83.8°$		
1.73 " " " 10 per cent HCl at 26.6°C.....................			$-78.3°$		
$[\alpha]_{Hgj}$ 3.46 per cent in H_2O at 15°C ...		$-98.3°$			
1.73 " " " 10 per cent HCl at 15°C.....................		$-89.2°$			
10 per cent in H_2O at 27°C.....				$-10.2°$	
5 " " " 10 per cent HCl at 27°C.....................				$-1.6°$	

English workers, but a different temperature (28.5 and 26.6°C. respectively instead of 15°C.). The room temperature was always too high to permit readings at 15°C. and we had no rotation tubes available which would permit the keeping of a constant lower temperature. We found, however, that in the determination of the

rotation of glutathione the temperature coefficient has a considerable influence on the result. This was shown by cooling the 3.46 per cent solution ($[\alpha]_{Hg}$ $-93.9°$ at 28.5°C.) to about 5°C. in a refrigeration room. When this solution was brought to the polarimeter room the solution with the same light, as soon as it could be read, gave a value of $-100.9°$ and then returned to $-93.9°$ after standing for some time at 28.5°C. The range of rotation between a temperature of 5–28.5°C. is therefore from $-100.9°$ to $-93.9°$. A similar temperature coefficient was found for cystine solutions by Andrews (1). The figures obtained with electric light are given for the convenience of workers who are not provided with a mercury light. These figures are considerably lower, as could be predicted.

As to the rotation of SH glutathione, it should be pointed out that Hunter and Eagles first called attention to the probability that this substance had "a very small negative rotation, or possibly a positive rotation." These workers dealt with an impure product. The figures in Table I for the rotation of the sample of SH glutathione were obtained with a product made according to the directions given in this paper, which should avoid partial oxidation. It is seen that this product has a small negative rotation in water ($-10.2°$) and a very slight negative rotation ($-1.6°$) in 10 per cent HCl. Other batches of SH glutathione showed a slightly different rotation, but of the same order of magnitude.

Absence of Cystine or Cysteine.—The samples of glutathione referred to in Table I were tested for the presence of free cystine or cysteine by the naphthoquinone test of Sullivan (9) and were found to be free from either of these substances. This test is of some value for testing the purity of samples of glutathione. The majority of our products gave a negative test, but a few of the early batches prepared by the original (Hopkins) method gave a curious purple coloration different from that produced by cystine and cysteine.

In view of the fact that Van Slyke's amino acid nitrogen estimation yields unreliable results in the case of cystine-containing substances, as was also found for glutathione by Hopkins, we dispensed with this determination. We also thought it superfluous to carry out a hydrolytic analysis which is never quantitative, as stated by Hopkins.

Finally, mention should be made of the heavy metal derivatives obtained from SH glutathione. Their exact constitution has not been determined. From the analysis of the copper compound, however, it is fairly certain that the copper substitutes the hydrogen of the SH group and that the copper is monovalent. This would indicate that part of the cupric salt used is reduced to cuprous salt with the simultaneous oxidation of part of the SH glutathione to S—S glutathione. We found that cuprous hydroxide also forms a compound with SH glutathione which appears to be of the same composition as that obtained with cupric salts. The second atom of Cu is probably attached to a carboxyl group. S—S glutathione does not give any precipitate with copper salts, as stated by Hopkins.

From the analysis and the color of the gold derivative it appears that the metal is in the aurous state and probably attached to sulfur. Auric salts are strong oxidizing agents and should easily oxidize part of the SH glutathione to S—S glutathione. This view would explain the small yield.

The analysis of the lead compound and the fact that S—S glutathione does not yield a precipitate when treated with lead acetate indicate that one valence of the lead is attached to sulfur and the other probably to a carboxyl group.

A few attempts to prepare a mercury compound of definite composition so far have failed. At all events the preparation of these heavy metal derivatives furnishes additional information for a proper understanding of the significance of glutathione in the chemical defense of the body against poisoning with certain heavy metals (Voegtlin, Johnson, and Dyer (10)).

SUMMARY.

1. The original method of Hopkins for the preparation of glutathione from yeast sometimes yields products which are impure.

2. A modified method is described in detail, which, if carefully followed, will yield a product having the same composition and properties as given by Hopkins for diglutaminyl-cystine. The previous claims of Hopkins as to the chemical nature of glutathione are therefore confirmed.

3. The optical rotation of pure SH glutathione was determined for the first time.

4. The copper, gold, and lead compounds of SH glutathione have been prepared.

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THE ABSORPTION AND UTILIZATION OF INULIN AS EVIDENCED BY GLYCOGEN FORMATION IN THE WHITE RAT.

By MABEL G. BODEY, HOWARD B. LEWIS, AND JOHN F. HUBER.

(From the Laboratory of Physiological Chemistry, Medical School, University of Michigan, Ann Arbor.)

It is generally agreed that no enzymes which can effect the hydrolysis of the polysaccharide inulin are secreted by the organs of the mammalian digestive tract (1, 2). That a slight hydrolysis by the hydrochloric acid of the gastric juice may occur in the human stomach, has been shown by Okey (3), who also observed the presence of an inulase in the feces. Fecal suspensions, as well as pure cultures of many intestinal bacteria (*Bacillus coli*, *Bacillus lactis*, etc.) were able to hydrolyze inulin (4) with the production of reducing sugars and organic acids (butyric, acetic, and lactic). It was thus evident that utilization of inulin in the higher organisms was dependent to a considerable extent on the activity of the microflora of the intestine.

The use of inulin or inulin-containing vegetables in the diet of the diabetic, suggested by Külz in 1874 (5), has been advocated by a school of German therapists (6). Goudberg (7), who observed increased respiratory quotients after the administration of inulin to diabetic patients, concluded that inulin was well utilized. When inulin was added to the diet of dogs fed meat only, a protein-sparing effect was noted by Shimizu (8), who interpreted this as evidence of the digestion and utilization of inulin in the digestive tract of the dog. The value of inulin in the dietary has not been recognized to any considerable extent in this country despite this evidence. Recently, however, Root and Baker (9) in the clinic of Joslin have reported favorable results following the use of Jerusalem artichokes by a group of diabetic patients, and considered that the beneficial results were due to the gradual liberation and slow absorption of fructose formed from the inulin, as evidenced by slight but definite increases in the level of blood sugar and in the respiratory quotient.

The question of the utilization of inulin has also been investigated from another standpoint, its behavior as a source of glycogen. Külz (10), Oppenheim (11), and von Mering (12) have maintained that after the administration of inulin to fasting rabbits, glycogen was formed, although the amount stored was less than after fructose feeding (11). On the other

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hand, Nakaseko (13) and Miura (14) considered that even under most favorable conditions, little glycogen was present in the liver after the ingestion of inulin, the rate of hydrolysis probably being too slow to permit of glycogen storage from the small amount of fructose absorbed. According to Lewis and Frankel (15), the administration of inulin *per os* did not increase the excretion of glucose in the phlorhizinized dog. Pflüger (16) and Mendel (1) after critically reviewing the literature concluded that inulin does not play an important rôle as a glycogen former.

In the work presented in this paper, the problem of inulin as a source of glycogen has been approached by a study of the glycogen content of the livers of young white rats after the oral administration of inulin. In addition the intestinal contents of these animals have been analyzed in order to determine the extent of absorption of the carbohydrate. Our results have demonstrated a slight, but, in our opinion, definite increase in the glycogen content of the liver after inulin feeding, thus indicating that in the white rat, under our experimental conditions, the hydrolysis of inulin by the acid of the gastric juice or by fecal inulase proceeded with sufficient rapidity to permit of some conversion of the product of hydrolysis after absorption to glycogen. We have no evidence as to the chemical nature of this source of glycogen, whether it be fructose, as seems most probable, or some organic acid, which can be converted to glycogen (*e.g.*, lactic acid), formed by the activity of the intestinal microflora (4).

<div align="center">EXPERIMENTAL.</div>

Young white rats, of 50 to 100 gm. weight for the most part, were fasted 24 to 48 hours as indicated in Tables II and III.[1] The experimental diets were then placed in the cages and the animals were allowed to consume the food at will for 48 hours (24 hours in a few later experiments). The food cups were removed and the amount of the food consumed was determined. The animals were then killed by chloroform, the livers were removed immediately, and glycogen determinations were carried out according to Pflüger. The glucose formed in the hydrolysis of the glycogen was estimated

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[1] Barbour, Chaikoff, Macleod, and Orr (17) have recently shown that more glycogen was present in the liver of the white rat in 48 hours than in 24 hours after withdrawal of food. In experiments carried out by us following the publication of their paper, a preliminary fasting period of 24 hours was used.

by the Folin-Wu method for blood sugar or by Somogyi's (18) modification of the method of Shaffer and Hartmann.

The alimentary canal was removed, the contents were washed into a mortar, and ground with a little sand. 10 cc. of sodium tungstate (10 per cent) were added and the contents of the mortar

TABLE I.

Recovery of Inulin and Fructose Added to Blood as Analyzed by Application of the Methods of Folin and Wu.

All results are expressed as mg. per 100 cc. of blood.

Carbohydrate added.	Reducing carbohydrate found.		Extra carbohydrate found.			
	Before acid hydrolysis.	After acid hydrolysis.	Before hydrolysis.		After hydrolysis.	
mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*
None.................	104.0	106.5				
"	104.5	106.0				
"	105.0	108.0				
"	105.0	106.5				
Average............	104.6	106.7				
Fructose 80..........	185.0	187.0				
" 80..........	186.0	187.5				
" 80..........	185.0	187.0				
" 80.........:	185.0	187.5				
Average............	185.2	187.2	80.6	100.7	80.5	100.6
Inulin 80*...........	121.0	189.5				
" 80	122.5	188.0				
" 80	122.0	188.0				
" 80	125.0	189.5				
Average............	122.6	188.7	18.0	22.5	82.0	92.3

* 80 mg. of inulin are equivalent to 88.8 mg. of fructose.

were transferred to a 100 cc. volumetric flask with the aid of water. 10 cc. of 0.66 N sulfuric acid were slowly added, the contents of the flask were diluted to the mark, shaken vigorously, allowed to stand for an hour, and were then filtered. In the filtrates, the reducing substances were determined directly by the colorimetric blood sugar method of Folin and Wu or by the Somogyi modifica-

tion (18) of the iodometric method of Shaffer and Hartmann. 5 cc. of the filtrate were placed in the tube in which the sugar determination was to be carried out, 0.1 cc. of 10 per cent hydrochloric acid was added, and the mixture was hydrolyzed for 10 minutes on the boiling water bath. The total reducing sugar after acid hydrolysis was then determined. Control experiments showed that under these conditions inulin was quantitatively hydrolyzed in the amounts present in our experiments. Inulin and fructose were also added to blood and the amounts of these sugars recovered before and after hydrolysis of the blood filtrates were determined by the Folin-Wu method. A typical experiment is presented in Table I. The results indicate that fructose is quantitatively recovered by this procedure. The acidity of the Folin-Wu filtrate (which is also obtained in our deproteinization of the intestinal contents) is, however, sufficiently great to effect a slight hydrolysis of the easily hydrolyzable inulin, between 20 and 25 per cent of the inulin added being recovered as a reducing sugar without the previous hydrolysis by hydrochloric acid. After hydrolysis, somewhat over 90 per cent of the inulin added was recovered.

An attempt was made to prepare a palatable carbohydrate-free diet, which would be eaten readily by the experimental animals. After several unsuccessful trials, a diet of butter oil (Osborne-Mendel (19)) to which 1 per cent of Vegex had been added was fed as the basal control diet. For the experimental diets mixtures of 50 parts of the butter oil, 50 parts of either fructose or inulin, and 1 part of Vegex were used. This food was eaten readily and was not wasted by scattering as were some of our earlier diets.

<div align="center">DISCUSSION.</div>

The results are summarized in Tables II and III. In our first series, eight rats fed the control fat diet had an average liver glycogen content of 0.29 per cent. This average figure is much increased by the inclusion of the figures for Rat 1a in which the unusually high figure of 0.72 per cent glycogen was obtained. With this exception, the variations are not marked, the glycogen contents varying from 0.11 to 0.38 per cent. These figures compare favorably with the values for liver glycogen obtained by Barbour, Chaikoff, Macleod, and Orr (17) in the white rat after brief periods

TABLE II.

Rat No.[*]	Weight.	Fasting period.	Feeding period.	Type of food.	Food intake.	Intestinal contents, reducing sugar.		Liver.		
						Before hydrolysis.	After hydrolysis.	Weight.	Glycogen content.	
	gm.	hrs.	hrs.		gm.	gm.	gm.	gm.	mg.	per cent
1a	109.5	48	48	Butter oil.	7.5	25.4	25.7	4.01	28.8	0.72
2a	81.6	48	48		7.3	12.5	12.9	2.36	5.2	0.22
3b	66.2	24	48		6.5	4.3	4.4	2.85	6.1	0.22
4b	66.5	24	48		6.3	2.7	2.7	2.82	10.6	0.38
5c	104.0	36	48		9.7	10.2	10.5	10.17	12.0	0.12
6c	79.5	36	48		6.8	8.0	8.2	4.03	11.2	0.28
7d	61.0	36	48		5.4	5.6	5.7	2.51	2.7	0.11
8d	78.4	36	48		5.9	5.7	5.8	3.44	10.9	0.32
Average.............................					6.9	9.3	9.5		10.9	0.29
9c	133.3	36	48	Butter oil and fructose.	11.8	20.1	20.5	7.72	122.4	1.59
10c	121.5	36	48		10.2	16.0	16.4	11.37	51.6	0.45
11d	61.3	36	48		8.5	104.9	106.7	3.53	61.6	1.75
12d	71.1	36	48		8.5	16.0	16.2	4.04	56.0	1.39
13d	64.5	36	48		8.5	16.0	16.2	3.42	61.6	1.80
Average.............................					9.5	34.6	35.2		70.6	1.39
14a	86.2	48	48	Butter oil and inulin.	5.8	41.7	262.8	3.04	20.7	0.68
15a	82.0	48	48		5.8	121.8	655.8	3.17	19.0	0.60
16a	76.1	48	48		5.8	86.2	395.6	2.73	31.0	1.13
17a	120.9	48	48		12.6	36.8	232.4	4.47	52.8	1.18
18a	150.5	48	48		14.9	22.0	190.3	5.92	37.3	0.63
19a	61.1	48	48		12.2	16.1	123.9	3.15	21.3	0.67
20b	64.0	24	48		11.6	22.9	128.3	3.28	22.7	0.69
21b	65.5	24	48		12.4	25.8	114.3	3.76	18.8	0.50
22b	63.5	24	48		11.2	22.5	88.9	3.20	23.3	0.73
23c	120.5	36	48		12.6	21.4	179.2	5.28	23.0	0.44
24c	142.0	36	48		11.9	26.2	183.6	9.56	37.9	0.40
25d	91.2	36	48		13.8	46.4	237.2	4.56	37.1	0.81
26d	53.0	36	48		10.1	18.2	120.5	2.43	15.4	0.63
27d	60.6	36	48		10.2	21.1	121.8	2.66	14.2	0.53
Average.............................					10.8	37.8	216.7		26.7	0.69

* The letters following the number of the rat indicate the experimental groups.

of fasting.   The glycogen content of the livers of the five rats fed
fructose was nearly 5 times as great as that of the controls, an aver-
age figure of 1.39 being obtained.   In this series, one liver showed
an abnormally low content of glycogen which lowers the average
considerably.   The average glycogen content of the livers of the
rats receiving inulin was more than double that of the animals on
the control (butter oil) diet (0.69 per cent as compared with 0.29
per cent).   In two experiments (Rats 16a and 17a) marked depo-
sition of glycogen was observed.   Expressed as mg. of liver glyco-
gen per 100 gm. of body weight, the average glycogen values of
our series were 14.8, 78.1, and 30.5 mg. for the animals fed butter
oil, butter oil and fructose, and butter oil and inulin respectively.

<div align="center">TABLE III.</div>

Rat No.	Weight.	Fasting period.	Feeding period.	Diet.	Food intake.	Liver.		
						Weight.	Glycogen.	
	gm.	*hrs.*	*hrs.*		*gm.*	*gm.*	*mg.*	*per cent*
1h	88.5	24	24	Butter oil.	2.6	3.12	2.3	0.07
2h	80.5	24	24	" "	3.4	3.14	2.1	0.07
3h	80.5	24	24	" " and fructose.	3.7	3.62	71.8	1.98
4h	92.0	24	24	" " " "	3.6	4.16	136.3	3.27
5h	95.5	24	24	" " " inulin.	6.1	4.20	70.5	1.68
6h	86.0	24	24	" " " "	5.4	3.47	29.2	0.84

It is of interest also to note that although the average total
food consumption was greater in those experiments in which the
rats were fed carbohydrate (9.5 gm. and 10.8 gm. in the experiments
with fructose and inulin respectively) than in the control experi-
ments (6.9 gm.), there was actually less butter oil consumed by
these animals since the carbohydrate diets contained only 50. per
cent of the butter oil.   If the glycerol of the fat were an important
factor in the formation of glycogen in our experiments, more
glycogen should have been formed in the control (butter oil) series
of animals.   It seems reasonable to assume then that any differ-
ences observed were due to the addition of carbohydrate to the
diet.

A consideration of the carbohydrates present in the intestinal

contents is also of interest. Relatively little carbohydrate was found in the control (butter oil) series, either before or after hydrolysis. With one exception absorption of the fructose was very complete and no significant amounts of additional carbohydrate which reduced alkaline copper solutions after hydrolysis were present. In the series of animals fed inulin, notable amounts of carbohydrate which reduced only after hydrolysis were present (*i.e.*, inulin). This is in accord with what might have been anticipated if inulin, as a polysaccharide, must be hydrolyzed before absorption and if hydrolysis occurs primarily through the agency of the bacterial flora which might be expected to vary in individual animals. We do not believe that the slightly increased amount of reducing carboydrate before hydrolysis is significant, since, as we have shown (Table I), the acidity of the Folin-Wu filtrate, which we employed, is sufficiently great to hydrolyze in part a readily split carbohydrate as inulin.

The results presented in Table III with another short series, in which the Somogyi (18) method was used to determine the glucose formed after hydrolysis of the glycogen, are similar to and even more striking than those already presented in Table II and discussed.

We believe that these results demonstrate a glycogen storage after inulin feeding under the conditions of our experiments. Whether the results obtained with pure inulin incorporated in a fat-rich diet such as we employed can be paralleled by the results of a series with inulin and the fructosans as they exist in the artichoke, is open to question. It seems possible that the high fat content of the food may have checked the activity of the flora by tending to prevent ready access to the inulin and thus have tended to prevent further decomposition of any fructose formed.

We have carried out a short series of experiments in which, under the conditions of fasting already outlined, freshly dug Jerusalem artichokes[2] were fed to white rats. The glycogen contents of the livers after feeding were less constant than in our

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[2] These experiments were carried out in April and May. At this season of the year, more reducing sugars and less inulin might be expected to be present than if the experiments had been made with artichokes dug in the fall or winter months. Compare the analytical findings of Root and Baker ((9) p. 128).

previous inulin series. The values obtained with a series of twelve rats were 0.04, 0.05, 0.11, 0.11, 0.32, 0.52, 0.58, 0.70, 0.71, 0.78, 1.13, and 1.14 per cent of glycogen. These values indicate a storage of glycogen in some cases. The experiments were complicated by the diarrhea which was an almost constant occurrence after the ingestion of such relatively large amounts (20 to 30 gm.) of raw artichokes as were eaten by our animals. This may be an important factor in the variable results obtained. Also it must be remembered that carbohydrates other than inulin are present in the tuber of the Jerusalem artichoke and that glycogen formation may result from these other carbohydrates.

SUMMARY.

Young white rats after fasting periods of 24 to 48 hours were fed a basal diet of butter oil, and experimental diets of butter oil plus fructose, and butter oil plus inulin. There was obtained no evidence of the formation of significant amounts of liver glycogen from the basal diet. A marked deposition of glycogen in the liver was observed after the addition of fructose to the basal diet. After the addition of inulin to the diet, although the effect was not as marked as with fructose, the glycogen content of the liver was clearly increased over that of the control animals on the basal (butter oil) diet.

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STUDIES ON GOSSYPOL.

I. THE PREPARATION, PURIFICATION, AND SOME OF THE PROPERTIES OF GOSSYPOL, THE TOXIC PRINCIPLE OF COTTONSEED.

By E. P. CLARK.*

(From the Food Research Division of the Chemical and Technological Research Branch of the Bureau of Chemistry and Soils, United States Department of Agriculture, Washington.)

The condition known as cottonseed injury, which often follows the incautious feeding of cottonseed meal to livestock, has been shown by Withers and Carruth (1) and by Schwartze and Alsberg (2) to be the toxic effect of a peculiar phenolic body known as gossypol.

Undoubtedly this substance was first prepared in a fairly pure condition by Marchlewski (3) who gave it its name. His preparation contained loosely bound acetic acid and had a melting point of 188°C. He reported analyses of a number of samples but was not able to establish with certainty a molecular formula, although he suggested $C_{13}H_{14}O_4$ or $C_{32}H_{34}O_{10}$. He made no crystalline derivatives of the material but showed that it was not a glucoside and in all probability contained no alkoxyl groups. He recorded some color reactions and its behavior toward certain reagents.

No further outstanding chemical work was published upon the subject until Carruth (4) communicated his interesting results in 1918. This investigator reported improvements in the preparation of gossypol, thus making possible more extended studies. A summary of his results is as follows: He found that gossypol was a phenolic substance, $C_{30}H_{30}O_9$ or $C_{30}H_{28}O_9$. When crystallized from acetic acid it contained 1 molecule of loosely bound acid which could be removed by dissolving the substance in ether, add-

ing water, and then removing the ether by evaporation.   The acetic acid remained in the water and could be titrated.   In this way a value near 532 was obtained for its molecular weight.   Determinations of this constant by physicochemical methods were not conclusive.   Values ranging from 300 to 595 resulted by the use of different methods and solvents.   When gossypol was dissolved in aniline, or when gossypol and aniline were dissolved in an appropriate solvent, a characteristic crystalline product was formed.   Carruth assumed the compound to have the formula of $C_{30}H_{28}O_9 \cdot 2C_6H_5NH_2$.   No carbon or hydrogen figures were given on this material, but those for nitrogen were reported from 3.97 to 4.84 per cent.   Crystalline acyl derivatives were not obtained, but analysis of the amorphous acetyl derivative indicated the presence of four acetyl groups.   When gossypol was heated to its decomposition point a new substance called B gossypol was formed.   This was obtained crystalline and apparently represented gossypol less 2 mols of water.

The above constitutes essentially all that is recorded concerning the chemistry of this interesting substance.   For this reason, and also because of the relation of gossypol to the so called cottonseed injury, a new chemical study of the substance has been undertaken.   The hope is entertained that the information thus obtained will be useful in the problem of detoxication of cottonseed meal.   The present communication is a preliminary report upon this work.   It deals with the preparation, purification, and analysis of gossypol as well as certain of its derivatives.

The data submitted herewith indicate that the molecular formula for gossypol is $C_{30}H_{30}O_8$.   This formula differs from Carruth's, but, in the light of the experience gained in the purification of the substance, it seems doubtful whether Carruth's material was entirely pure.   As recorded in the experimental part, a reddish brown impurity was associated with the gossypol, when prepared by Carruth's method, and was removed only with great difficulty by a large number of solvents suitable for its recrystallization.   A combination of solvents was finally found, however, which gave the desired result, but again difficulty was encountered in obtaining the material free from the solvent used in its purification.   In a number of cases, a small indefinite amount of solvent remained with the crystals.   This could not be removed in a 0.1 mm. vacuum

over both sulfuric acid and potassium hydroxide and at temperatures which would insure the integrity of the substance. Several solvents, however, were found which, under certain conditions, gave material having uniform composition. The criterion used to verify this was to obtain such preparations from different solvents as gave check analyses. When this was accomplished, carbon and hydrogen figures were obtained which indicated the formula given above. Various derivatives, prepared from the purified gossypol, also gave values upon analysis which checked the formula indicated. Among these were the so called "acetate;" *i.e.*, gossypol + 1 molecule of acetic acid, anhydrogossypol (Carruth's B gossypol), the aniline condensation product, the dioxime, and a hexaacetyl derivative.

The gossypol obtained in this work was a bright canary-yellow crystalline substance with a melting point of 214°C. (corrected). In solution no optical activity was exhibited. Molecular weight determinations by the camphor method of Rast (5) gave results of the order required for the formula suggested. Also, the acetic acid in the so called acetate was found to be 10.51 per cent, which gave a molecular weight of 511 for gossypol, assuming 1 mol of the latter to be associated with 1 mol of acetic acid. Gossypol was soluble in ether and acetone, but only sparingly soluble in other common organic solvents. It was insoluble in water, but dissolved readily in dilute ammonia and sodium carbonate. It was soluble in aqueous fixed alkalies; but its solution, with an excess of alkali, slowly decomposed, giving a brown solution which gradually became a beautiful purple. However, when potassium hydroxide was added to an aqueous suspension of finely ground gossypol, in amounts slightly more than enough to cause complete solution, the excess alkali could be titrated and the acid value of the substance was thus determined. Assuming that 2 equivalents of potassium hydroxide were used, molecular weight values of 505 and 507 were obtained. Its solution in concentrated sulfuric acid had a deep scarlet color. Upon diluting this acid liquid with water, the gossypol was thrown out unchanged. It was readily soluble in saturated methyl alcoholic hydrochloric acid. The color of the solution was first brown which gradually turned deep green and finally intense blue. When heated to its melting point, gossypol lost 2 mols of water, producing a new crystalline body, anhydrogossypol,

$C_{30}H_{26}O_6$. This substance possessed quite different solubilities, but still retained the characteristic color reactions of the original material. Oximation of gossypol produced a colorless dioxime. This seemed to indicate that the chromophore is a quinone group. As stated by Carruth, gossypol combined with aniline to form a deep orange-colored crystalline compound. This substance was found to be a condensation product formed by 1 mol of gossypol uniting with 2 mols of aniline, with the elimination of 2 mols of water. Its formula was found to be $C_{42}H_{40}N_2O_6$, and it is probably a Schiff base type of compound.

Acetylation of gossypol was carried out with acetic anhydride, with use of sodium acetate and also pyridine as catalysts. With sodium acetate, by employing the conventional proportions of reagents (1 part of acetate, 4 parts of substance, and 16 parts of acetic anhydride) and by boiling the mixture for 10 minutes, a non-crystallizable product was obtained which analyzed for 30.8 per cent acetyl. But when a large excess of the reagents was used and the boiling continued for 2 hours, a material resulted, about 10 per cent of which was obtained as beautiful crystalline plates. Acetylation in pyridine solution gave this same product but the yield was better. This substance, when analyzed for acetyl by Perkin's method (6) for O-acyl compounds, gave from 24.9 to 25.9 per cent acetyl. These figures agreed quite well for a tetra-acetyl gossypol (25.07 per cent). Carbon values, however, were those of a hexaacetyl body. It was then found that if Perkin's method (6) for N-acyl compounds was used, the percentage of acetyl required for a hexaacetyl derivative was obtained. The crystalline material was therefore a hexaacetyl gossypol, indicating that in the parent substance there were six hydroxyl groups. Thus all the oxygen in gossypol has been accounted for. 2 atoms are present as carbonyl groups, as was shown by the formation of a dioxime, and 6 are present as hydroxyl groups. It is to be emphasized, though, that two of these hydroxyl groups behave quite differently from the remaining four. This was manifest by the drastic treatment necessary to remove the last two acetyl groups, as just mentioned, and also by the fact that two hydroxyl groups are capable of being titrated as an acid.

Finally it is desired to call attention to some toxicity tests in which rats were used as experimental animals. These tests were

conducted solely for the purpose of checking the toxicity of several preparations during the course of their purification. In this work, however, certain observations were made which seemed to be of sufficient interest to warrant reporting.

White rats, whose average weight was 190 gm., were injected intraperitoneally with cottonseed oil solutions of the various

TABLE I.

Toxicity Tests Made upon Different Preparations of Gossypol.

The material, dissolved in cottonseed oil, was given intraperitoneally to white rats, the average weight of each being 190 gm.

Date.	Material used.	Dose.	No. of rats used.	No. of deaths result-ing.	Time in days until death.	Remarks.
1927		*mg. per kg.*				
Feb. 9	Crude gossypol acetate.	25	1	0	0	
		50	2	2	2, 2	Acute.
		100	1	1	1	"
May 4	Gossypol recrys-tallized from chloroform.	25	2	2	6, 13	All but 13 day animal acute.
		50	2	2	2, 2	
		75	2	2	1, 1	
		100	2	2	1, 1	
May 17	Gossypol ʃtwice recrystallized from ether and petroleum ether.	20	2	2	7, 10	All acute type.
		30	2	2	3, 5	
		40	1	1	3	
		50	1	1	1	
June 20	Purified gossy-pol recrystal-lized from ether (analyt-ically pure).	10	6	2	17, 18	2 day and 3 day ani-mals acute, all others chronic.
		15	6	5	10, 11, 12, 12, 13	
		20	6	5	2, 3, 9, 10, 10	

preparations. There was no apparent difference between the toxicity of the crude gossypol acetate and the analytically pure gossypol. Rats receiving doses of 20 mg. or more per kilo of body weight died in from 20 hours to 13 days. Of those receiving from 10 to 20 mg. per kilo, 50 per cent survived; but in the remaining, deaths occurred in from 2 to 18 days. Though the percentage of deaths at 15 mg. per kilo equals that at 20 mg. per kilo, death

resulting from the latter dosage was the result of acute poisoning, whereas death at 15 mg. per kilo was the result of chronic poisoning.

Postmortem findings in the acute type of poisoning were oily droplets and plaques in the peritoneum, congested lungs, hemorrhagic condition of all the viscera, and cardiac dilatation. In the chronic type of poisoning the outstanding indication was intestinal impaction. The cause of delayed death was stated by Dr. Formad, pathologist of the Bureau of Animal Industry, "to be probably due to a paralyzing effect upon the sympathetic nervous system." Dr. Formad further stated that "it seemed probable that there was a tonic spasm of the sphincter ani accompanied by general decreased intestinal tonus which led to decreased rate of elimination of feces and finally to an intestinal impaction. This latter, in turn, produced pressure upon the diaphragm which was transmitted to the heart and lungs and served as a probable cause of death."

In connection with the toxicity work just mentioned, assistance was obtained from Dr. James C. Munch and Miss Georgiana S. Gittinger of the Pharmacology Laboratory of this bureau. It is a pleasure for the writer to express here his thanks to these workers for their kind cooperation.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Preparation of Gossypol.*</div>

The gossypol used in this work was prepared by the method of Carruth (4). Ground cottonseed meats were first freed from oil by extraction with petroleum ether. 3 kilos of the air-dried material thus obtained were percolated, at a moderate rate, with ethyl ether until 7.5 liters of extract were obtained. Two such lots were combined and the ether was distilled until the residue had a volume of 1 liter. This was filtered and further concentrated, under reduced pressure, to 400 cc. To this solution, 300 cc. of acetic acid were added and the mixture was set aside to crystallize. After 2 hours, the so called gossypol acetate which had formed was freed from the mother liquor, washed with acetic acid, and dried. 80 kilos of ground seed gave 385 gm. of crystals, a yield of 0.48 per cent. The gossypol acetate consisted of somewhat imperfectly shaped plates which melted at a temperature between 184–187°C. Its solution in ether had a brownish red color, owing to an impurity

which was difficult to remove by recrystallization. It was found, however, that the material could be purified by liberating the loosely bound acetic acid and crystallizing the free gossypol from appropriate solvents. To accomplish this the following procedure was adopted. 50 gm. of the crude crystals were suspended in 400 cc. of ether. Approximately 4 volumes of water were added, and the mixture was stirred until all the substance dissolved in the ether layer. The contents of the beaker were then heated on the steam bath until the ether evaporated and the water had a temperature of about 60°C. Under this treatment the gossypol became a porous mass and could be broken up. To facilitate this, it was cooled by adding crushed ice. The gossypol was then filtered from the water containing the acetic acid, washed with cold water, and dried. The yield was 45 gm.

To purify the crude product it was dissolved in ether and filtered. 2 volumes of petroleum ether (b.p. 35–70°) were added to the filtrate, and the solution was concentrated, under reduced pressure, until crystals began to form. The liquid was removed from the flask, and ½ volume of petroleum ether was added which caused crystallization to take place very rapidly. The yield was generally 70 per cent. The mother liquors were concentrated, as described above, until crystallization began. Upon standing some time, a second crop of crystals was obtained, amounting to 15 per cent of the original material. The latter was treated as new material and was further purified by repeating the above process. The resulting mother liquors were quite darkly colored. They were evaporated, *in vacuo*, to dryness, dissolved in ether, and acetic acid was added. In this way some crude acetate was always recovered which was reworked.

The first crop of crystals was recrystallized a second time to free them completely from the red impurity. The resulting product, as well as its solution in ether, had a bright canary-yellow color. It was not used for analysis, however, as it contained an indefinite amount of the solvent employed in its crystallization, and this could not be readily removed. It was therefore recrystallized from different media which gave uniform preparations of definite composition.[1]

[1] Of the methods to be described, only the last two are practical from the preparative standpoint. The ether method is recommended.

The first solvent used was dilute alcohol.   A boiling saturated solution of the purified gossypol in 95 per cent alcohol was diluted with water until a slight turbidity occurred.   The liquid was set aside and was occasionally stirred during crystallization.   The product obtained had a distinct green color but gradually turned an orange color during several days' exposure to the air.   This air-dried material consisted of irregularly shaped fractured plates which began to soften at 190°C. and melted with decomposition at 199° (corrected).   A molecular weight determination made by the camphor method of Rast (5) gave the following results:

12.3 mg. of substance dissolved in 136.8 mg. of camphor, $\Delta = 6.3°$.

$$M = \frac{(40,000)\ (12.3)}{(136.8)\ (6.3)} = 571.$$

0.1982 gm. substance gave 0.1067 gm. H_2O and 0.5045 gm. CO_2.

For $C_{30}H_{30}O_8$.   Calculated.   M 518.4.   C 69.48 per cent, H 5.83 per cent
           Found.   " 571.   " 69.44 "   "   " 6.01 "   "

Its optical properties were found to be as follows:[2] pale yellow plates, in part obliquely rhombic in outline.   The substance dissolved in organic liquids, so its refractive indices were determined by immersion in solutions of mercuric potassium iodide in glycerol. $\alpha = 1.605$; $\beta = 1.740$; γ was much greater than the highest liquid available, but it was determined as approximately 1.83 by immersion in molten mixtures of piperine with antimony-arsenic iodide. For identification purposes the lowest of these values was found to be the most useful, as it was shown by the majority of the grains in one crystallographic direction.

Another portion of the purified gossypol was recrystallized from ether and methanol in the following manner.   4 volumes of methanol were added to a filtered ethereal solution of gossypol. The solvents were removed by distillation, under reduced pressure, until crystallization began.   The contents of the flask were removed

[2] The optical properties of this and of several of the compounds, to be described later, were kindly determined by Dr. Edgar T. Wherry of this bureau, and Mr. George L. Keenan of the Food, Drug and Insecticide Administration.   The writer wishes at this time to express his thanks to Dr. Wherry and Mr. Keenan for their valuable cooperation.

and crystallization was allowed to proceed to completion. The preparation obtained in this way had a somewhat greenish color but became bright yellow upon exposure to the air during a week.

In the latter condition, the substance consisted of irregularly shaped fractured plates which began to darken and contract at 205°C. and melted with decomposition at 214° (corrected). Its optical properties were found to be the same as those recorded for the previous preparation. Molecular weight determination by the Rast method gave the following.

10.7 mg. of substance dissolved in 110 mg. of camphor, $\Delta = 6.8°$.

$$M = \frac{(40,000)\ (10.7)}{(110)\ (6.8)} = 572.$$

I. 0.1092 gm. substance gave 0.0585 gm. H_2O and 0.2778 gm. CO_2.
II. 0.1973 " " " 0.1059 " " " 0.5032 " "

For $C_{30}H_{30}O_8$. Calculated. M 518.4. C 69.48 per cent, H 5.83 per cent.
Found. I. " 572. " 69.40 " " " 5.99 " " .
II. " 69.58 " " " 6.01 " "

A third method of obtaining solvent-free gossypol was to crystallize the purified substance from ether. This was done by stirring on a steam bath an ethereal solution of the purified material with 4 volumes of water until the ether had evaporated and the water had a temperature of approximately 60°C. Upon cooling, the gossypol was obtained as a bright yellow porous mass which consisted of very small irregularly shaped plates. It began to soften and contract at 209° and melted with decomposition at 214° (corrected). The optical properties of this preparation could not be determined, owing to the minuteness and physical state of the crystals. Molecular weight determinations were made by quickly dissolving a weighed amount of substance in an excess of 0.1 N KOH, and titrating the excess alkali with phenolphthalein as an indicator.

I. 0.3373 gm. material used 13.3 cc. 0.1 N KOH.
II. 0.4169 " " " 16.5 " 0.1 " "
Molecular weight found (assuming 1 mol of gossypol requires 2 mols of KOH). I, 507; II, 505.
I. 0.2055 gm. substance gave 0.1091 gm. H_2O and 0.5229 gm. CO_2.
II. 0.1958 " " " 0.1040 " " " 0.4979 " "
Found. I. C 69.42 per cent, H 5.94 per cent.
II. " 69.37 " " " 5.94 " "

So Called Acetate Prepared from Purified Gossypol.

As this compound is important in the preparation and identification of gossypol, a sample was made from purified starting material in order that accurate data concerning it could be determined. The substance was obtained by adding acetic acid to an ethereal solution of gossypol whereupon crystallization began immediately. It consisted of bright yellow broad plates with notched ends, occasionally rhombs, which began to soften at 180°C. and melted with decomposition at 189–190°C. (corrected). The acetic acid content of the substance was determined by dissolving the material in ether, adding water, and then removing the ether by evaporation on the steam bath. When the gossypol was separated by filtration, the acetic acid in the aqueous filtrate was titrated.

0.4996 gm. acetate required 8.75 cc. 0.1 N KOH.
I. 0.1074 gm. acetate gave 0.0585 gm. H_2O and 0.2600 gm. CO_2.
II. 0.1929 "   "   " 0.1043 "   "   " 0.4668 "   "
For $C_{30}H_{30}O_8 \cdot CH_3COOH$.

Calculated. CH_3COOH 10.38%, C 66.41%, H 5.92%.
Found. I.   "   10.51%, " 66.04%, " 6.09%.
        II.   " 66.02%, " 6.05%.

Its optical properties were found to be as follows: bright yellow plates, often obliquely rhombic. Oily immersion media were applicable for this substance. $\alpha = 1.550; \beta = 1.750; \gamma$ was higher than the highest liquid available, but it was determined approximately as 1.84 in piperine iodide melts. As in the case of pure gossypol, the lowest index was the most useful for identification purposes.

Anhydrogossypol.

Gossypol in $\frac{1}{2}$ gm. portions, contained in medium sized test-tubes, was heated with stirring in an oil bath to 215°C. As soon as a uniform mass was obtained and effervescence ceased, it was removed from the bath and cooled. The melt was quickly dissolved in ether and removed from the test-tube. Anhydrogossypol began to crystallize immediately and the process was complete in a few minutes. The compound was filtered, washed with ether, and dried. The yield was 45 per cent of theory. Purification was

accomplished by dissolving it in a large volume of dry ether and slowly evaporating this solution on the steam bath until crystallization began. The product consisted of orange-colored rods which softened and partly melted at 229–230°C., then melted competely to a black liquid quite sharply at 268°C. (corrected). It was very sparingly soluble in common organic solvents, but possessed the color reactions of gossypol.

I. 0.2019 gm. substance gave 0.0994 gm. H_2O and 0.5510 gm. CO_2.
II. 0.1410 " " " 0.0698 " " " 0.3842 " "
For $C_{30}H_{26}O_6$. Calculated. C 74.66 per cent, H 5.43 per cent.
Found. I. " 74.45 " " " 5.51 " "
II. " 74.34 " " " 5.54 " "

Its optical properties were found to be as follows: brownish yellow rods with oblique pyramidal terminations. $\alpha = 1.560$; β and γ were much higher than 1.75, the highest liquid available. In addition to the α value, which was shown lengthwise of the rods, the peculiar red and green polarization colors shown between crossed nicols were characteristic of this substance.

Dianiline Gossypol.

This substance was conveniently prepared by dissolving gossypol in 10 times its weight of boiling aniline. On cooling the solution, dianiline gossypol separated as a beautiful orange-colored crystalline mass. The crystals were freed from the mother liquor and were thoroughly washed with ether. The yield was practically quantitative. It was purified by recrystallization from benzene. A portion of the crude material was dissolved in boiling benzene, and the solution filtered and concentrated, under slightly diminished pressure, to about ⅓ its original volume. The substance crystallized immediately upon removing the concentrate from the flask and allowing it to cool. When heated rapidly its melting point was 302–303°C. with decomposition (corrected). In ordinary light it consisted of yellow rods with all the refractive indices greater than 1.75 and accordingly indeterminate. Between crossed nicols it showed parallel extinction and anomalous blue and green polarization colors owing to peculiar dispersion. This may be of use in recognizing the substance. Molecular weight·

determinations by the camphor method of Rast gave the following values:

I. 19.9 mg. dissolved in 195.3 mg. of camphor, $\Delta = 6°$.

$$M = \frac{(40,000)\ (19.9)}{(195.3)\ (6.0)} = 680.$$

II. 16.9 mg. dissolved in 182.3 mg. of camphor, $\Delta = 5.7°$.

$$M = \frac{(40,000)\ (16.9)}{(182.3)\ (5.7)} = 650.$$

I. 52.9 mg. substance required 4.67 cc. $N/30$ acid (Kjeldahl).[3]
II. 51.3 " " " 4.55 " $N/30$ " "
I. 0.1040 gm. " gave 0.0565 gm. H_2O and 0.2869 gm. CO_2.
II. 0.1202 " " " 0.0665 " " " 0.3328 " "

For $C_{42}H_{40}N_2O_6$.
Calculated. M 668.5. C 75.43 per cent, H 6.03 per cent, N 4.19 per cent.
Found. I. " 680. " 75.26 " " " 6.08 " " " 4.12 " "
II. " 650. " 75.53 " " " 6.19 " " " 4.14 " "

Gossypol Dioxime.

30 cc. of an alcoholic solution of hydroxylamine, made by exactly neutralizing with sodium hydroxide the hydrochloric acid in 3 gm. of hydroxylamine hydrochloride, were added to a suspension of 5 gm. of gossypol in 50 cc. of alcohol. The solution was warmed and stirred until all the gossypol was dissolved. The solution was then heated to boiling and set aside during the night to crystallize. The oxime was washed with alcohol, then with water, again with alcohol, and then dried. The yield was 4 gm. It was recrystallized by dissolving 1 gm. in 125 cc. of boiling alcohol, filtering, and adding an equal volume of water. Crystallization began at once

[3] Attention is called at this point to the necessity of using mercury as a catalyst in the Kjeldahl nitrogen determinations upon this substance. When copper was used as a catalyst, low values for nitrogen, ranging from 3.6 to 3.8 per cent (depending upon the period of digestion) were obtained. In some cases the digestion was prolonged 6 hours after the solution cleared. The low nitrogen figures upon this compound reported by Carruth and by Schwartze are accounted for by the fact that copper was used as a catalyst by them in their determinations (personal communications).

and was complete within an hour. The yield was 85 per cent of theory. The material consisted of thick rhomboidal plates which began to darken and contract around 225°C. but did not melt at 315°C. It had a faintly yellowish green color, but under the microscope the crystals were colorless.

I. 49.9 mg. substance required 5.28 cc. N/30 acid (Kjeldahl).
II. 50.6 "   "   "   5.35 " N/30 "   "
I. 88.3 "   "   gave 48.0 mg. H_2O and 210.8 mg. CO_2.
II. 93.5 "   "   "   51.3 "   "   " 223.9 "   "
For $C_{30}H_{32}N_2O_6$.
    Calculated. C 65.66 per cent, H 5.88 per cent, N 5.11 per cent.
    Found. I. " 65.13 "   "   " 6.08 "   "   " 4.94 "   "
           II. " 65.33 "   "   " 6.14 "   "   " 4.94 "   "

Acetyl Gossypol.

A mixture of 1 gm. of gossypol, 0.25 gm. of anhydrous sodium acetate, and 4 cc. of acetic anhydride were boiled under a reflux for 10 minutes. The resulting solution was diluted with an equal volume of acetic acid, cooled, and poured into 300 cc. of water. A light cream-colored product was precipitated which was freed from the mother liquor, washed with water, and dried.

0.4030 gm. substance required 28.9 cc. 0.1 N KOH (Perkin's O-acyl method).
            Acetyl found. 30.86 per cent.

3 gm. of gossypol acetate, 40 cc. of acetic anhydride, and 1.5 gm. of sodium acetate were boiled for 2 hours, after which the solution was treated in the manner described above. Yield, 4 gm. This material was dissolved in 75 cc. of boiling methanol. The next day a small amount of a crystalline material had separated. This was dissolved in boiling ethyl acetate, filtered, and 2 volumes of methanol were added. The solution was concentrated, under reduced pressure, until crystals began to form. After several hours the crystalline product was collected, washed with methanol, and dried. Yield, 0.4 gm. The material consisted of thin plates, nearly rectangular in outline and practically colorless. It began to soften and darken at a temperature of 255°C., and melted with decomposition at 276-277°C. (corrected). $\alpha = 1.515$; $\beta = 1.580$; $\gamma = 1.645$. The highest index was best adapted for the identifi-

cation of the substance. It was shown in one crystallographic direction by the majority of the plates.

I. 0.4280 gm. substance required 24.76 cc. 0.1 N KOH (Perkin's O-acyl method).

II. 0.2507 gm. substance required 14.9 cc. 0.1 N KOH (Perkin's O-acyl method).

III. 0.3024 gm. substance required 23.05 cc. 0.1 N KOH (Perkin's N-acyl method).

Acetyl found. I, 24.88 per cent; II, 25.56 per cent; III, 32.8 per cent.

I. 90.3 mg. substance gave 45.1 mg. H_2O and 216.0 mg. CO_2.

II. 138.3 "   "   " 70.5 "   "   " 331.1 "   "

For $C_{30}H_{24}O_8(CH_3CO)_6$.

Calculated. Acetyl 33.5 per cent, C 65.44 per cent, H 5.50 per cent.

Found. I. Acetyl(III) 32.8%, C 65.26%, H 5.59%.

II.   " 65.31%, " 5.70%.

The acetylation of gossypol in pyridine solution gave the same compound as above but the yield was better. This procedure was carried out as follows: A solution of 5 gm. of gossypol in 30 cc. of pyridine was treated with 10 cc. of acetic anhydride. The mixture was heated to its boiling point and was then allowed to stand for $2\frac{1}{2}$ hours. Upon pouring the reaction mixture into water 8.8 gm. of cream-colored material were obtained. The crystalline hexa-acetate was obtained by dissolving the substance in methanol and allowing it to crystallize during a day. Upon recrystallization, as outlined in the above experiment, 1.6 gm. of pure acetyl body were obtained.

The optical properties and melting point were the same as given above.

A molecular weight determination by the camphor method gave the following:

14.4 mg. of substance dissolved in 184.7 mg. of camphor, $\Delta = 4°C$.

$$M = \frac{(40,000)\ (14.4)}{(184.7)\ (4)} = 780.$$

For $C_{42}H_{46}O_{14}$. Calculated. $M = 770.5$.

SUMMARY.

1. Gossypol, the toxic principle of cottonseed, has been prepared in a state of analytical purity, and its molecular formula

has been ascertained to be $C_{30}H_{30}O_8$. Certain properties of this purified material have been recorded.

2. Analytical data, including optical properties in certain cases, have also been obtained upon several derivatives of gossypol. Those included are the so called acetate, anhydrogossypol, the dianilide, the dioxime, and the hexaacetyl derivative.

3. The dianilide, which is important because of its use in the quantitative determination of gossypol, has *not* been found to be a salt of gossypol (1 mol of gossypol + 2 mols of aniline), as Carruth has suggested, but a condensation product formed by 1 mol of gossypol condensing with 2 mols of aniline, with the elimination of 2 mols of water.

4. Of the 8 oxygen atoms in gossypol, 2 have been shown to be present as carbonyl groups, whereas the remaining 6 have been shown to be present as hydroxyl groups. Two of these hydroxyl groups behave differently from the remaining four, being much more acidic and requiring drastic treatment for the hydrolysis of their acetyl derivative.

5. A short report concerning some interesting physiological effects of gossypol upon rats has been made.

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CHANGES IN THE OXYGEN CAPACITY OF THE BLOOD PIGMENT OF RABBITS FOLLOWING THE ADMINISTRATION OF NITROBENZENE.

By BARBARA B. STIMSON.*

(*From the Department of Physiology, College of Physicians and Surgeons, Columbia University, New York.*)

INTRODUCTION.

The changes that occur in the blood pigment of animals after the administration of nitrobenzene have been the subject of some discussion. Dittrich (2) and Ray and Stimson (3) working on dogs found spectroscopic evidence of methemoglobin after giving sublethal doses of the drug. Van Slyke and Vollmund (6) in their experiments on rabbits found an anemia but no change in the character of the blood pigment after large amounts of nitrobenzene. Because there seemed to be a definite time factor in the appearance and disappearance of methemoglobin after the administration of the drug (3), the question arose as to whether or not the last named workers might have taken the blood samples too early or too late to find the change in the blood pigment. To answer this question the following work was undertaken.

Methods.

Rabbits were used throughout the experiment. The nitrobenzene, 0.1 cc. per kilo of body weight, was given in about 5 cc. of olive oil by stomach tube and was retained in every case. No evidences of toxicity were observed within the period during which the blood was taken. Blood samples were drawn immediately before and at varying intervals after the administration of the drug (see Table I). All samples were taken from the ear

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* National Research Council Fellow in Medicine.

veins and were oxalated. Van Slyke's hydrosulfite-CO method (5) was used in making the analyses, and at the same time spectrophotometric readings were made. The technique employed was similar to that used by the writer in a preceding investigation (4).

TABLE I.

Amount of Non-Oxygen-Bearing Pigment in Per Cent of Total Hemoglobin.

Rabbit No.		Hrs. after medication.						
	Initial.	1st	2nd	3rd	4th	5th	6th	7th
		per cent	per cent	per cent	per cent	per cent	per cent	per cent
268	0				7.16			
278	0		0			2.41		
275	0			0		4.38		
291	0				5.11		2.20	
102	0				3.69		12.5	
108	0					3.18		2.47
119	0	0		0		14.70		0
68	0		0		2.38		0	
154	0		0		8.02		0	
168	0	0		3.73		2.29		0
145	0		4.30		5.35		0	
Second dose 4 wks. after first.								
278	0			0?		5.05		
275	0	9.05		8.95		0		0
291	0		0?		0		11.78	
268	0					0		0
From 18 to 60 days after splenectomy.								
132	0	0		13.55		6.25		3.96
127	0	13.30		11.86		4.24		0
156	0	0		4.24		2.40		0
140	0		5.12		10.80			
108	0	13.68		0		0		0

Results.

A summary of the results of the experiment is given in Table I. Eleven normal rabbits were used; four were given a second dose 4 weeks after the first; five were given the drug from 18 to 60 days after splenectomy. In all cases the total blood pigment

and the oxygen-carrying hemoglobin agreed within experimental error in the initial sample; *i.e.*, that taken before the nitrobenzene was given. Within 7 hours afterward all cases except one showed a loss of oxygen capacity of from 2.2 to 14.7 per cent of the total pigment. The one exception (Rabbit 268, second dose) showed no loss in oxygen-bearing pigment, but as the sample was not taken until the 5th hour after the drug was given an early loss may have been missed.

Spectrophotometric examinations of the blood samples showed at no time any evidence of methemoglobin. The ratios obtained for the light absorption at two wave-lengths (Å. = 5600 and Å. = 5400) were similar to those given for oxyhemoglobin in the literature (1).

DISCUSSION.

From the above data it would seem that nitrobenzene when put into the stomachs of rabbits causes the formation of a hemoglobin derivative which is incapable of carrying oxygen but which may be reduced by hydrosulfite to active hemoglobin. This inactive pigment is, therefore, probably an oxidation product of hemoglobin similar to but not the same as methemoglobin as it does not give the characteristic spectrophotometric picture of the latter.

The variations in the amount and in the time of appearance of the hemoglobin compound may in part be explained by differences in absorption through the gastrointestinal walls of the individual rabbits. The contents of the stomach at the time of administration of the drug may also play a part.

Though not enough experiments were performed to justify any definite conclusions, it is interesting to note that splenectomy seemed to increase the amount of inactive pigment and to hasten its formation. This again suggests the possibility of a reducing action of the spleen already postulated (Ray and Stimson (3), Stimson (4)).

SUMMARY.

The results of this paper may be summarized as follows: Nitrobenzene when given to rabbits by stomach tube produced

in the circulating blood a non-oxygen-carrying pigment varying in amount from 2.2 to 14.7 per cent of the total blood pigment. This inactive pigment did not give the spectrophotometric picture characteristic of methemoglobin. It appeared within 7 hours after the administration of the drug.

The writer wishes to make grateful acknowledgment of the kind advice and criticism of Dr. H. B. Williams and Dr. E. L. Scott and of the able technical assistance of Mr. Louis Dotti.

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THE UTILIZATION OF INTRAVENOUS SODIUM r-LACTATE.*

I. EXCRETION BY KIDNEYS AND INTESTINES.

By HAROLD A. ABRAMSON AND PHILIP EGGLETON.

(From the Institute of Physiology, University College, London, England.)

INTRODUCTION.

The work of Meyerhof (1919, 1920, 1921) concerning the recovery process in isolated muscle preparations, and the calculations made by Hill (1924) during the recovery process in man, have led to the conclusion that in the recovery process after exercise about four-fifths of the lactic acid produced during exercise is resynthesized to glycogen. The remainder, or an equivalent of carbohydrate, is believed to be oxidized. The rôle of the lactate ion in the intact animal seems to have been studied without obtaining much direct chemical evidence as to its influence on normal carbohydrate metabolism.

Araki (1894) administered sodium lactate subcutaneously to dogs, and found that after doses of about 5 gm. only a negligible portion was excreted. After showing that during carbon monoxide poisoning the lactate is totally excreted, he concluded that the lactate is completely oxidized in the normal dog, making an assumption that the poison had merely reduced the oxygen supply of the tissues. This interpretation was disputed by Minkowski (1893), who showed that following liver extirpation lactate in the urine was increased.

Janssen and Jost (1925), after demonstrating that intravenous injection of sodium lactate into the intact dog produced no increase in muscular glycogen and carbohydrate, assumed that the lactic acid introduced into the intact animal was synthesized to glycogen in the liver. Elias and Schubert (1918) had earlier reached the same conclusions.

With the object of obtaining direct chemical evidence as to the distribution and ultimate fate of injected lactate the authors have administered sodium r-lactate to a number (85) of dogs. The experiments fall into two groups: (1) (a) By determining the

amount and nature of the excess base in the urine following injection, and (b) by following changes of the CO_2 content of the blood; (2) (a) by following changes in the lactate content of the blood, urine, liver, muscle, and intestinal contents; (b) by studying changes in the blood sugar level, and in the glycogen content of the liver and muscle tissue; (c) by measuring oxygen consumption.

The first problem was to determine the magnitude of the errors arising from excretion of lactic acid in the urine and intestinal tract. These data form the basis of this first communication.

<div align="center">EXPERIMENTAL.</div>

<div align="center">*Methods.*</div>

In the first series of experiments morphine and scopolamine anesthesia was used. Two dogs were studied without anesthesia, and the remaining experiments, which form the basis of this report, were performed under amytal (isoamylethylbarbituric acid) Lilly (Deuel, 1924–1925; Page, 1923–1924). It does not prevent glycogen synthesis by the liver (see Paper III of this series).

Bitches that had been fasted at least 1 day were always used. As soon as each animal was brought to the laboratory it was given a purgative dose of calomel and kept on a water diet. The urine was obtained by catheterization of the bladder, and blood was obtained from the jugular veins. Lactate was injected into the external saphenous veins of the hind leg. The temperature was regulated approximately to 38°C. The water balance of the animal was maintained by repeated subcutaneous injections of warm tap water. Saline was not used because of its influence on acid-base equilibrium (see Paper II of this series).

Racemic lactic acid (sp.gr. 1.21) was diluted and boiled under a reflux for 8 to 10 hours to hydrolyze the lactide present. The lactate solution for injection had a pH of about 7.4, the strength varying between 2 and 10 per cent. The amounts injected varied between 0.5 and 4 gm. per kilo.

A modification of the method of Clausen (1922) was used throughout. The distillation process had an efficiency of 89 to 92 per cent.

The preparation of the test solution for examination was as follows:

Urine.—Diluted and freed from possible traces of sugar by the Van Slyke technique (copper sulfate and lime (Van Slyke, 1917)). Estimation was made on appropriate quantity of filtrate.

Intestinal Washings.—The pyloric end of the stomach was tied to the warm water tap and the whole intestine washed through
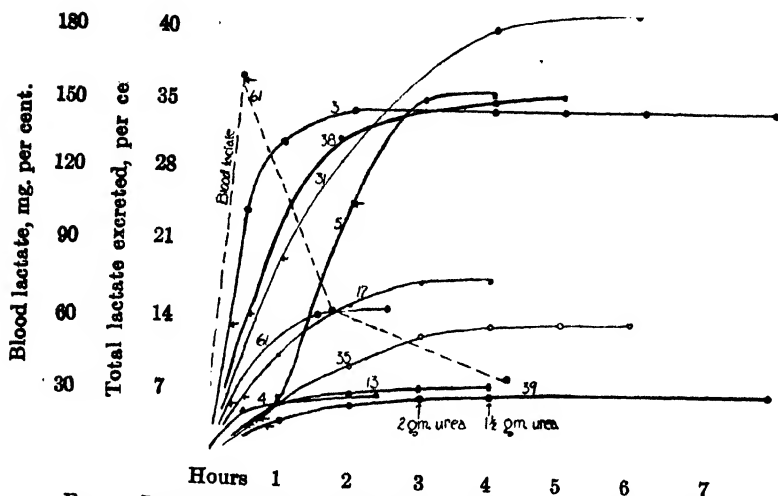
Fig. 1. Between 2 and 3 hours after intravenous injection of sodium r-lactate excretion is practically complete under the conditions described in the text. When the injection period is less than 40 minutes the excretion is practically complete within 2 hours. Urea diuresis has no effect on quantity output. Arrows point to end of injection. The typical drop of blood lactate is also demonstrated by the dotted lines. Numbers on curves refer to number of experiment.

with about a liter of water. If the bile was to be included, the contents of the gallbladder were squeezed into the duodenum beforehand. The solution was filtered and treated as under "Urine."

Blood.—Deproteinized by the Schenck method (Hirsch-Kauffmann, 1924). The solution was cleared of sugar by the Van Slyke technique.

TABLE I.

See Figs. 1 and 2 for further data.

Experiment No.	Anesthesia.	Weight of dog.	Dose.	Total excretion.	
		kg.	*gm.*	*gm.*	*per cent of dose*
2	M. S.		2.6 per kg.		25
3	"		0.84 " "		34
4	"		0.4 " "		6
5	"		10.2	3.67	36
6	"		2.0	0.15	7.5
7	None.		8		25
8	"		16		34
13	E. A.	6.5	12.7 in 78 cc.	0.77	7.6
17	A.	6.5	6.1 " 100 "	1.0	18
28	"	10	30 " 198 "	6.7	27
29	"	5	15 " 300 "	5.8	38
31	"	7.5	16.4 " 300 "	7.26	44
35	"	6.5	10 " 200 "	1.36	13.6
38	"	8	8.6 " 144 "	3.1	36
39	"	8.5	5.4 " 216 "	0.38	7

M. S. = morphine and scopolamine.

E. A. = amytal with ether induction.

A. = amytal.

It is worth while to note the following additional data available. Experiment 29. Blood lactate 443 mg. per cent at end of injection; liver lactate 407 mg. per cent at end of injection; 2½ hrs. later, when animal was killed, it had dropped in the blood to 273 mg. per cent. Experiment 31. Blood lactate 72.5 mg. per cent 6 hours after beginning of injection. Experiment 38. Blood lactate 63.5 mg. per cent at end of experiment. Experiment 17. See curve for blood lactate levels (Fig. 2). Experiment 61. See curve for blood lactate levels (Fig. 2).

TABLE II.

Experiment No.	Urine excretion in first 3 hrs.	Lactic acid excretion.
	cc.	*per cent of dose*
31	510	42
28	445	27
29	413	38
5	316	36
38	252	35
35	214	13.5
17	208	18
13	83	7.6
39	59	7

The largest percentage excretion is concomitant with greatest urine output, suggesting that the degree of diuresis is conditioned by the lactate excretion (provided the water balance of the animal is maintained).

Results.

Urine.—The excretion of lactate in the urine is practically completed in 3 hours after the finish of the injection (Figs. 1 and 2, Tables I and II).   With doses of sodium *r*-lactate similar to those described, it may be assumed that under these conditions lactate excretion is complete in this time.   There is a rapid drop in the concentration of lactic acid in the blood synchronous with
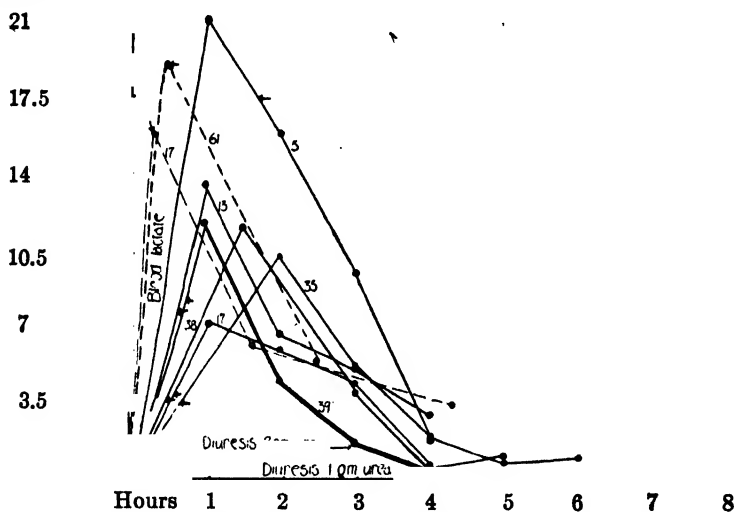
Fig. 2. Concomitant with the drop in concentration of lactic acid in the urine and the completion of the excretion, there is a fall in blood lactic acid. Janssen and Jost (1925) found this fall in blood lactate to be more rapid after sodium *d*-lactate.   Arrows indicate end of injection.   Dotted lines represent blood lactate of concentrations one-tenth those of ordinates. Numbers on curves refer to number of experiment.

the drop in its concentration in the urine.   The most concentrated urines (1 to 2 per cent lactate ion) are excreted during the 1st hour following the peak of lactate concentration in the blood (Fig. 2).   The normal lactate titer of dog urine is comparatively so small (20 mg. per cent) that it may be neglected.

Two preliminary experiments with sodium *l*-lactate (Irvine, 1906) have given excretion curves similar to those of the racemic

salt.   The excretion is of the same order of magnitude (20 to 30 per cent) in 6 hours.   The *l*-lactate used gave almost theoretical analytical values and optical rotation (two specimens).   One of these experiments was complicated by hematuria.

Since the lactate excretion is invariably less than 50 per cent of the lactate injected as sodium lactate (sometimes less than 10 per cent), it seems probable that the *l*-lactate radicle is utilized without difficulty.

It is difficult to state definitely which of the two isomers of lactic acid was utilized with the greatest ease in the experiments

TABLE III.

Experiment No.	Weight of dog.	Dose of lactic acid injected as sodium salt.	Intestinal lactic acid.	Remarks.
	kg.	*gm.*	*mg.*	
11	8.5	0	50	Control. Stomach to rectum. Bile lactic acid 60 mg. per cent.
12	9.75	0		Bile lactic acid 50 mg. per cent.
14	11	0	65	Control.
13	6.5	10	120	4 hrs. after injection. Bile included.
17	6.5	6.1	60	3 hrs. after injection.
19	13.5	15.2	37	3 " " " Small intestine only.
31	7.5	16.4	117	6½ hrs. after injection. Bile included.
32	6.0	30.0	119	Dog killed immediately after injection.

Following intravenous Na *r*-lactate injection, the loss of lactic acid through the intestinal tract may be neglected.

where the racemic salt was used.   More than 90 per cent of the injected racemic mixture is retained in certain experiments.   The preliminary *l*-lactate experiments and the analysis of the salt excreted in *r*-lactate experiments (see following paragraph) suggest that the intact animal utilizes both isomers with almost equal facility.   Meyerhof and Lohmann (1926) find, however, that isolated tissues utilize *d*-lactate faster than *l*-lactate.   The differences these authors found for isolated tissues are far greater than could be deduced from our experiments on the intact animal where

the oxidative processes are so much better regulated and are favored by blood and lymph circulation.

In one experiment (No. 28) the excreted lactate yielded a zinc salt which analyzed as the racemic salt.   The animal weighed 10 kilos and received 2 gm. per hour for 4 hours.   It excreted 6.7 gm. during the injection.   The analysis of salt was as follows:

	Water of crystallization. per cent	Optical activity. Hg green line. degrees
Inactive salt	18.2	0
l-Salt	12.9	+9
Found	18.6	+1

Intestinal Tract.—As Table III shows, the amount of lactic acid excreted into the intestine is negligible under our experimental conditions.   We have not directly estimated the quantity of lactate excreted in the bile, but our results show that if any such excretion occurs it is followed by reabsorption.   Hence the intestinal tract as a source of error may be neglected.   Note also that two specimens of normal gallbladder bile treated by the Van Slyke desugarizing technique gave a lactic acid titer of 50 and 60 mg. per cent.

SUMMARY AND CONCLUSIONS.

Sodium *r*-lactate has been injected intravenously into fasted intact anesthetized dogs and the following results observed.

1. According to the dosage and the rapidity of the injection, lactate may be excreted unchanged in the urine to the extent of about 7 to 40 per cent of the amount injected.   The excretion is practically completed within 3 hours after the completion of the injection.

2. The excreted lactate appears to be mainly racemic.

3. There is little or no excretion of lactate into the intestinal tract.

4. The fall in concentration of blood lactic acid is synchronous with the fall in urinary lactate concentration.   The kidney was able to concentrate sodium lactate about tenfold.

On the strength of the evidence submitted in this paper, we shall assume for future work: (1) that the urine collected up to the end of the 3rd hour after completion of the injection of sodium

r-lactate contains practically all the lactate radicle that will be rejected by the animal; (2) that under our experimental conditions both isomers of lactic acid are utilized by the dog.

We would like to thank Professor A. V. Hill for his kind interest and suggestions, and Dr. M. G. Eggleton for doing part of the analytical work.

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THE UTILIZATION OF INTRAVENOUS SODIUM *r*-LACTATE.*

II. CHANGES IN ACID-BASE EQUILIBRIUM AS EVIDENCE OF UTILIZATION.

BY HAROLD A. ABRAMSON AND PHILIP EGGLETON.

(*From the Institute of Physiology, University College, London, England.*)

INTRODUCTION.

It has been shown in the preceding communication (Abramson and Eggleton, 1927) that within 3 hours after the intravenous injection of sodium *r*-lactate approximately 10 to 40 per cent of the lactate injected is excreted in the urine. The remainder, 60 to 90 per cent, is retained by the body and probably utilized.[1]

The only other possibility is storage as sodium *r*-lactate in a particular tissue or group of tissues. Although this is most unlikely, it must be so proven by suitable evidence. It is the purpose of this communication to show by further evidence that: (1) utilization of the injected lactate does take place, and (2) incidental to this utilization there are marked changes in acid-base equilibrium.

These changes have been studied by measurement of: (*a*) CO_2 content of blood; (*b*) fixed CO_2 excreted in the urine; (*c*) titratable alkalinity of urine; (*d*) estimation of phosphate in urine.

If the lactate ion be utilized,

$$\text{Na lactate} \rightleftharpoons \text{Na}^+ + (\text{lactate}^- \text{ utilized})$$
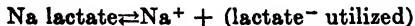
(whether the utilization be oxidation or carbohydrate synthesis), there will be left in the blood stream and tissues an excess of base. This excess of sodium ion should increase the bicarbonate content

[1] See the recent paper of Reigel, C., *J. Biol. Chem.*, 1927, lxxiv, 135.

of the blood, and with this rise in blood alkali produce a flushing out of alkaline salts through the kidneys.   This is precisely what has been found experimentally.   The details are presented subsequently.

Methods.

The preparation of the bitches was as previously described. Note that lactic acid was always administered as the sodium salt.

Blood carbon dioxide was determined by the method of Van Slyke (1917).   Determinations were done in duplicate and corrected for blood volume by hemoglobin determinations in a Duboscq colorimeter.   The CO_2 content of the urine was also estimated by Van Slyke's method.   When mixtures of bicarbonate and basic phosphate were titrated with methyl red, 90 per cent of the bicarbonate and about 103 per cent of the phosphate could be determined at the end-point of this indicator (pH 4 to 6).

For this reason the urines were titrated with methyl red when urinary "excess base" was desired.   It will later appear that the base excreted in the urine is mostly in the form of bicarbonate with relatively little phosphate.   Hence the values obtained by this method are only slightly below theoretical.   The range of titration converts Na_2HPO_4 to NaH_2PO_4 and $NaHCO_3$ to the sodium salt of the acid used in the titration.   By this method and by considering the base excreted as its equivalent, it was possible to estimate the minimum of lactate utilized.

Phosphate was determined by a modification of the method of Bell and Doisy (1920).

Results.

Blood.—(See Table I and Fig 1.)   Amytal anesthesia produces no appreciable change in the CO_2 content of the blood (confirming Deuel).   Synchronous with the intravenous injection of sodium lactate there is a marked and sudden rise in the CO_2 content of the blood.   There is no cyanosis and there is a definitely increased oxygen consumption.   This rise, therefore, cannot be attributed to incomplete ventilation.

Note that in Experiment 51, the value observed was as high as

TABLE I.

CO_2 Content of Blood Following Intravenous Injection of Sodium r-Lactate (Volumes Per Cent).

Size of dose is given as lactic acid. Ether-induced amytal anesthesia employed. With the exception of Experiments 14 and 15 all values given have been corrected for blood volume. Time is after beginning of injection.

Experiment No.	Weight of dog.	Dose.	Injection time.	Before injection.	CO_2 content of blood, vol. per cent.							
					½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	8 hrs.
	kg.	gm.	min.									
14	11	0		70	65.2			69.5				
15	12	10	30	65	77	85.2				67.2	71.5	
19	13.5	15.2	25	62		83	79	78				
27	10.5	10.5	25		66–75 (20 min.)	72.5 (1 hr., 20 min.)	69	70.5 (3 hrs., 20 min.)				
29	5.0	15.0	120	53			96	(3 hrs., 30 min.)				
31	7.5	16.4	55	58	86	82	86					
35	6.5	10.0	40	57.5	82				81		73	
51	8.5	31.8	160	58				110 (2 hrs., 40 min.)	49			75.5
61	6.5	4.2	18	55	77 (20 min.)	61 (1 hr., 30 min.)		50				

110 volumes per cent.   Generally speaking, the greater the dose
the higher was the level of blood CO_2 reached.   This relationship
is merely suggested by the data presented, for from the few curves
no definite statement may be made.   After the peak of the rise is
reached the blood CO_2 level falls toward normal gradually.   But
following large doses the CO_2 remains above normal within the
experimental periods described.
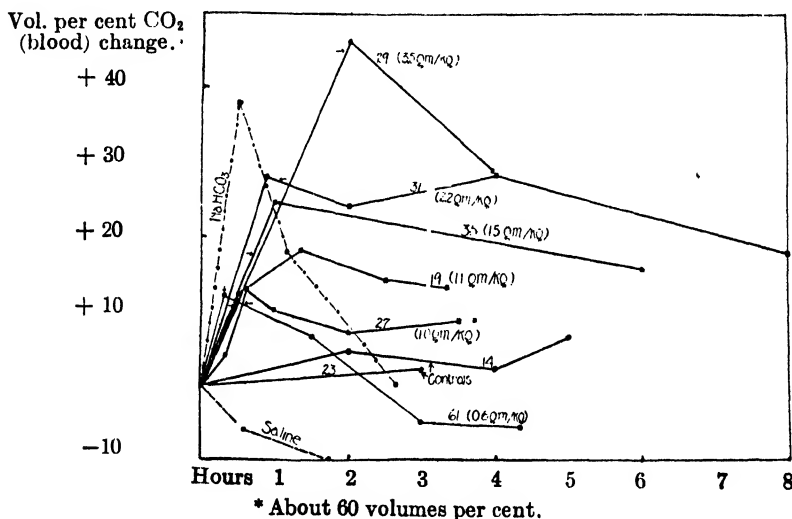
Fig. 1. These curves represent the differences between normal and
experimental values after administration of $NaHCO_3$, NaCl, and sodium
r-lactate intravenously.   Note (1) the relative acidosis produced by in-
travenous saline, (2) the sudden drop toward normal after $NaHCO_3$, and
(3) the persistent alkalosis encountered during utilization of sodium
r-lactate administered in large doses.   In Experiment 61, however, the
blood CO_2 dropped below normal.

In Experiment 61, however, the dose was $\frac{2}{3}$ of a gm. per kilo,
and here the blood CO_2 fell below normal.   The fact that there is
a constant excess of base in the blood permits of urine titration in
order to obtain minimum values for lactate utilization, if we take
base excreted as equivalent to lactate utilized.   For the titratable
alkalinity to represent minimum utilization, the blood CO_2 must
remain above normal during the period of urine collection (see
influence of intravenous NaCl on acid-base equilibrium below).

The curve given by intravenous sodium bicarbonate is quite different from the curve resulting from lactate injection. The drop of the blood CO_2 to normal following bicarbonate administration is accomplished within a relatively short time. The origin of this difference may be found in the slow utilization of lactate and the relatively normal blood volume found after lactate excretion. Hence little water is left to excrete the bases. Intravenous saline produces no rise in the CO_2 content of the blood; in fact, a slight drop has always been observed incidental to saline diuresis. This was reported previously by Hendrix and Calvin (1925).

To summarize then, the sudden and rapid rise in the blood CO_2 following the injection of sodium r-lactate seems to indicate rapid utilization of the injected material with the subsequent excretion through the kidneys of some of the liberated base. The administration of equivalent amounts of inorganic salts of sodium does not produce the same effect.

Urinary Excretion of Base.

As stated previously, a minimum value for lactate utilization may be estimated by studying the base excretion in the urine. This is only true if the blood CO_2 be above normal during the period of urine collection. Control experiments show that an intravenous injection of NaCl may produce a relatively large amount of urinary base including bicarbonate.

The concomitant drop in blood bases has been mentioned in the preceding section. The following experiment (No. 47) demonstrates this effect of salt diuresis on the titratable alkalinity of the urine.

Experiment 47.—Bitch, fasted 2 days; weight 7½ kilos. Ether-induced amytal anesthesia was employed. H_2SO_4 equivalent to sodium salt of amytal was injected subcutaneously. Presence of carbonates was noted by degree of effervescence. Amytal was injected at 10.50 a.m.; 200 cc. of distilled water subcutaneously. At 1.05 p.m., 200 cc. of 2 per cent saline were injected intravenously in 25 minutes. Urine was collected at end of 6 hours. Carbonates present throughout experiment.

Total base excreted following injection =   15.4 cc. N H_2SO_4.
    "   urine   "   "   "   = 143   "
Base expressed as lactic acid utilized (if so calculated) = 1.65 gm.

Urine volume and base excreted always seemed to be independent of one another.

When small doses of sodium _r_-lactate are given, even though there be a primary rise in the CO_2 content of the blood incidental to the diuresis, the level of CO_2 in the blood may fall below normal. In these cases, as the following experiment (No. 48 B) demonstrates, a false value may be obtained for lactate utilization determined through base excretion.

Experiment 48 B.—3 gm. of sodium _r_-lactate were injected.   Urine was collected at end of 8 hours.

Total base excreted = 27.4 cc. N H_2SO_4.

Base excreted expressed as lactate utilized = 2.9 gm.

Blood CO_2 at beginning of experiment, 54 volumes per cent; at end of experiment, 49.5 volumes per cent.

Even though a theoretical value has here been obtained for lactate utilized, in the light of the final low blood CO_2 it cannot be accepted as representing lactate utilized.

However, with large doses, as in the following experiment (No. 35), the blood CO_2 remains above normal.   Hence a minimum estimate of the rate of lactate utilization may be made, since the base excretion occurs while the blood CO_2 remains persistently high.

Experiment 35.—Bitch; weight 6½ kilos.   Amytal anesthesia employed. Urine was collected under paraffin.   Bladder was not washed out at end of experiment.   Results are given in Table II.

Lactic acid excreted = 1.8 gm.

Total base   "   = 4.3 " lactic acid.

Approximately seven-ninths of this base was in the form of bicarbonate.   The minimum value for the lactate utilized during this period as determined above is then:

$$\frac{4.3}{\underset{\text{(injected)}}{10.0} - \underset{\text{(excreted)}}{1.8}} = 52 \text{ per cent.}$$

If the soft tissues are in equilibrium with the blood in respect to its fixed CO_2 content (73 per cent) the remainder of the lactate retained in this experiment may be easily accounted for by this excess of base throughout the body.   That such an equilibrium does exist is rendered probable by the following calculation.

1. CO_2 excreted in urine between 1.30–7.30 p.m. = 34 cc. N H_2SO_4.

2. Fall in blood CO_2 level over same period (assuming $\frac{6.5}{13}$ liters or 500 cc. blood volume) = 2.2 cc. N H_2SO_4.

3. Therefore, the excreted bases would have only been about 6 per cent of the value obtained had they been derived only from the blood.

TABLE II.

Time.	Blood CO_2.	Urine volume.	Titratable base excretion in cc. N H_2SO_4.	CO_2 in urine in cc. N H_2SO_4, expressed as bicarbonate.	Difference between titratable base and CO_2 in cc. N H_2SO_4.	Rate of base excretion per min. in cc. N H_2SO_4.	Rate of excretion of CO_2 in cc. N H_2SO_4 per min.	Rate of excretion of difference between titratable base and CO_2 in cc. N H_2SO_4 per min.
	vol. per cent	*cc.*						
12.30 p.m.	57.5							
12.40 " Injected 10 gm. lactic acid in 200 cc. solution.								
1.20 p.m. injection finished.								
12.40–1.30 p.m.		45	2.7	1.3	1.4	0.054	0.026	0.022
1.35 p.m.	82.6							
1.30–3.15 p.m.		113	15.5	12.2	3.3	0.15	0.11	0.032
3.15–4.40 "		57	12.1	10.0	2.1	0.14	0.13	0.028
4.40–6.30 "		41	11.1	8.2	2.9	0.10	0.074	0.026
6.30–7.30 "		20	4.8	3.6	1.2	0.08	0.060	0.020
7.30 p.m.	73.0							
Totals.................		276	46.2	35.3	10.9			

As the excretion of lactate diminishes, the rate of excretion of base increases. This is particularly noted in experiments where concentrated urine lactates are found; that is, while lactate is being excreted a relatively small amount of urinary base appears. In Experiment 56, 27.4 gm. of lactic acid were administered (as Na r-lactate). In the course of 2 hours 6.65 gm. of lactic acid were found in the urine. By a method to be discussed in the next paper, it was calculated at least 8.6 gm. had been utilized. But,

only 16.6 cc. of N H_2SO_4 were used to titrate the base excreted, and this represents less than a quarter of lactate probably utilized.

The lactate excretion in the urine drops rapidly towards normal but, as in Experiment 60 (Fig. 2), the base excretion persists much longer. This is in agreement with the persisting alkalosis. The major portion of excreted base is in the form of bicarbonate, with

Mg. lactate cc. per min.	Titers of urine cc. N H_2SO_4 per min.
6.0	0.24
5.0	0.20
4.0	0.16
3.0	0.12
2.0	0.08
1.0	0.04

Hours   1   2
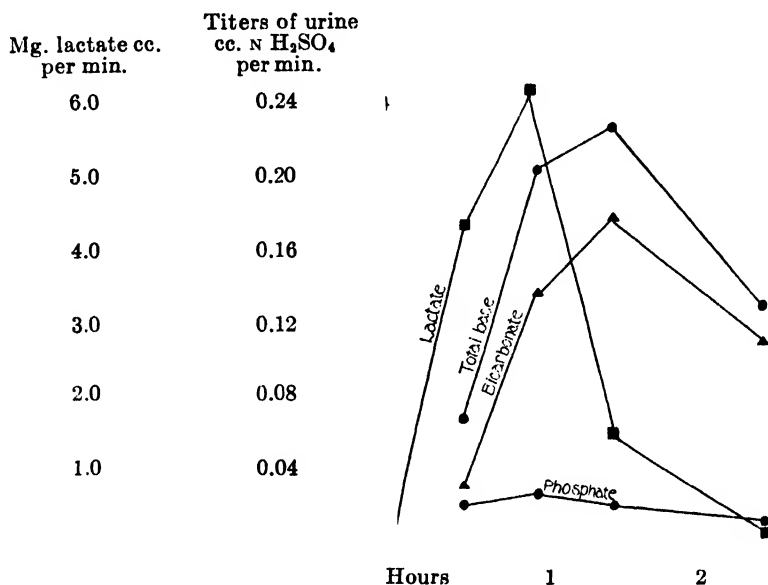
FIG. 2. The lactate excretion in the urine drops rapidly toward normal but the base excretion persists. This is to be expected because of the gradually diminishing but nevertheless persistent alkalemia. These curves demonstrate that the major portion of excreted base is in the form of bicarbonate with a small and relatively constant rate of phosphate excretion. The sum of the equivalents of bicarbonate and basic phosphate is approximately equal to the base excreted. Injection time 30 minutes. The blood lactate was 360 mg. per cent at the end of the injection. 2 hours later although the lactate in the urine had diminished considerably it was still twice normal, 113 mg. per cent.

a small and relatively constant phosphate excretion. The sum of the CO_2 (expressed as bicarbonate) and the basic phosphate excreted is approximately equal to the total base excreted (see Fig. 2). This relatively slight phosphate loss is particularly important in consideration of the glycogen synthesis studies reported in the succeeding paper.

SUMMARY.

Following the intravenous injection of sodium r-lactate:

1. There is a variable rise in the CO_2 content of the blood, which cannot be attributed to incomplete ventilation.

2. This rise persists for some hours after urinary lactate excretion is complete.

3. The rise in CO_2 content of the blood seems to be dependent upon the size of the dose of lactate administered.

4. Concomitant with the rise and fall in blood base there is an excretion of "excess base" in the urine.

5. Under suitable conditions, this "excess base" may represent the minimum of lactate utilized.

6. The "excess base" is mainly in the form of sodium bicarbonate. The rate of phosphate excretion is relatively constant.

7. Saline diuresis produces a lowering in the blood bases and an excretion of bases in the urine. This is a source of error which must be considered when estimating lactate utilized by base excreted.

CONCLUSIONS.

Incidental to the utilization of intravenously injected sodium r-lactate there is a profound alteration of acid-base equilibrium. These changes, which produce a high alkalemia, may influence carbohydrate synthesis, and at present prevent significant measurements of respiratory quotient to determine the nature of the substances burned during lactate utilization.

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THE UTILIZATION OF INTRAVENOUS SODIUM r-LACTATE.*

III. GLYCOGEN SYNTHESIS BY THE LIVER. BLOOD SUGAR. OXYGEN CONSUMPTION.

By HAROLD A. ABRAMSON, M. GRACE EGGLETON, AND PHILIP EGGLETON.

(From the Institute of Physiology, University College, London, England.)

INTRODUCTION.

This work was started primarily because it was hoped that some comparison might be drawn between the chemical changes occurring to sodium lactate produced during muscular exercise in the tissues and distributed to the blood and other tissues, and its reverse, the utilization of injected sodium lactate. That there is a basis for this comparison has been shown chiefly by Meyerhof (1926) and his coworkers.

This group has demonstrated that there are processes taking place in the isolated muscle and liver tissue of the mammal which enable synthesis of carbohydrate to take place from added sodium lactate,—a process similar to the carbohydrate synthesis following lactic acid production in stimulated isolated frog muscle. An objection may be raised that the alkalosis produced in the intact animal may influence—perhaps hinder—glycogen synthesis, and hence make intact animal experiments incomparable. In rat tissues suspended in phosphate-buffered Ringer's solution (pH 7.4) containing sodium lactate, carbohydrate synthesis was observed by Meyerhof. The pH of the solutions used by Meyerhof at the end of the experiment was at least 7.4, because the free base liberated incidental to the utilization of the lactate ion retains CO_2 with the formation of $NaHCO_3$. In the experiments of Meyerhof where KOH was used to absorb CO_2 the analogy with experiments on intact animals is even more direct. The CO_2 absorbed by the alkali leaves the system in the same way that the CO_2 is blown off in the dog.

The volume of buffering fluid is not given in the protocols.   The proportion of isotonic phosphate to Ringer's solution was 1:20. The method of Warburg (1923, 1924, 1925) used by Meyerhof necessitates a total volume of 3 to 8 cc.   Meyerhof, however, notes that when serum was used as the suspending fluid the pH at the beginning of the experiment was 8.0 and at the end might be 8.5.

At all events it is clear that carbohydrate synthesis taking place in the isolated mammalian tissue experiments of Meyerhof occurs in a definitely alkaline medium, and that during the process of the synthesis the reaction becomes, if anything, more alkaline.   Meyerhof has also shown that the isolated tissues utilize sodium d-lactate much more rapidly than the levo isomer.   The first paper of this series has pointed out that the wide differences noted by Meyerhof could not be deduced for intact dogs, which retain from 60 to 90 per cent of injected sodium r-lactate.

The importance of obtaining further data concerning the fate of lactate ion introduced into the intact animal is emphasized by the researches of Hill (1924) and his coworkers.   These authors conclude from their studies of oxygen consumption, CO_2 retention, and lactic acid formation after exercise in man, that the proportion of lactic acid burned to that resynthesized to carbohydrate (1:5) during the recovery period of man is about the same as that found by Meyerhof for the isolated frog muscle.

The study of the changes in muscle glycogen and carbohydrate following intravenous sodium r- and d-lactate into dogs (with spinal cord section for analgesia and quiet) has been carefully studied by Janssen and Jost.   Confirming earlier work by Elias and Schubert who perfused the hind limb arteries of dogs, Janssen and Jost found no change in muscle glycogen or total carbohydrate.   Nor did they find an increase in oxygen consumption by the muscles—thus helping to confirm the fact that under their experimental conditions there was a negligible (if any) synthesis of carbohydrate.

Both Janssen and Jost and Elias and Schubert concluded, however, that the lactate which rapidly disappeared from the blood was in great part synthesized by the liver to glycogen.   This previous work on muscle glycogen has been confirmed by the authors of this communication in two experiments.   And so, on the

basis of this confirmed finding of lack of muscle glycogen synthesis, it will be assumed in the remainder of this work that intravenous sodium lactate does not produce a demonstrable glycogen synthesis in the skeletal muscles of dogs.

Before proceeding to the experimental data obtained concerning glycogen synthesis, etc., a brief review of the literature pertaining to oxidation and storage of glucose and lactate by the liver in acidosis and alkalosis will serve to elucidate in further detail the problem that has been presented.

REVIEW OF LITERATURE.

In 1875 Luchsinger showed that the surviving liver perfused with glucose increased its glycogen content from 0.6 to 1.3 per cent in 1¾ hours. It was not until 1903 that his work was confirmed when Grube also demonstrated glycogen synthesis in the isolated turtle liver. During the previous decades Weiske and Flechsig (1889) had shown that 60 gm. of lactic acid administered as calcium lactate had the same sparing effect on the protein metabolism of sheep as an equivalent quantity of glucose. Araki believed that lactic acid was completely oxidized (1894). He showed that small subcutaneous doses of sodium r-lactate were almost completely retained by dogs but if oxidative processes were hindered by CO poisoning, the salt was completely excreted in the urine. His view was disputed by Minkowski (1893) who believed that the injury to the liver incidental to the poisoning had disturbed the total metabolism as well as the metabolism of the liver. That lactic acid could have its source from carbohydrates in the organism was first shown directly by Embden in 1905. He demonstrated that percolation with blood of a surviving liver rich in glycogen resulted in the appearance of lactic acid in the circulated fluid. If the liver was glycogen-free and the percolating fluid rich in dextrose, there also resulted considerable lactic acid formation. But if the liver and blood were both relatively carbohydrate-free a small production of lactic acid ensued. During this period attempts had been made by Embden and Salomon (1905) to demonstrate glucose formation from lactate in the diabetic animal. It was not until 1906, however, that the well controlled experiments of Mandel and Lusk conclusively proved that the phlorhizinized dog converts sodium r-lactate at least partially to glucose. In the same paper they pointed out that lactic acid disappears from the blood and urine in phosphorus poisoning when phlorhizin poisoning is induced. On the basis of this they believed the liver synthesized the lactic acid produced from the cleavage and denitrogenization of protein to dextrose, before its distribution to the tissues.

The first attempt to study the effect of reaction on glycogenesis seems to have been made by Pavy and Bywaters (1910–11). These authors found that the injection of dilute sodium carbonate into the liver of an anesthetized cat hindered the transformation of glycogen into glucose.

Neglecting the variations of glycogen content of the tortoise liver found by Schöndorff and Grebe (1911) and later confirmed by Noble and Macleod (1923–24), Parnas and Baer (1912) concluded from two experiments on the isolated tortoise liver that glycogen synthesis occurred during perfusion with sodium lactate.   The mean rise of their results is close to the limits of variability of glycogen content in different lobes of the liver as reported by Macleod (1926).   That the conclusion of Parnas and Baer may have been erroneous is supported still further by the work of Barrenscheen (1914). This author found that glycogen was produced by the surviving livers of rabbits and dogs during glucose perfusion.   Under the same conditions, sodium and ammonium lactate produced not a rise, but a drop in liver glycogen.   If, however, the livers were obtained from phlorhizinized dogs a rise in the sugar content of the perfusing blood indicated that glucose synthesis from lactate had taken place.   At about the same time the dispute concerning the ability of the liver of the intact animal to synthesize glycogen from glucose that had not first passed through the intestinal wall, was settled.   Ishimori (1913) and Freund and Popper (1912) showed that glycogen synthesis by the liver followed the intravenous injection of glucose into dogs and rabbits.

The work of Elias (1913) and Elias and Kolb (1913) started a long line of researches to determine the influence of hydrogen ion concentration on glycogenesis by the liver.   Elias found that relatively small quantities of acids administered through the stomach (70 cc. of 0.25 N HCl) to rabbits can cause glycogen to disappear from the liver in large quantities with accompanying glycosuria and hyperglycemia.   Although perfusion of tortoise livers with dextrose resulted in glycogen formation, a drop in liver glycogen occurred if acid was added to the perfusion mixture.   In the later paper with Kolb, Elias reported the prevention of the glycosuria occurring in the hunger diabetes of dogs.   Administration of alkali reduced the acidosis and simultaneously prevented or reduced the glycosuria.   This was further evidence that acidosis prevents the normal storage of glycogen.

The fact that acidosis hindered and alkalosis aided glycogen synthesis by the liver was shown indirectly by Macleod (1915–16).   Following the report of hypoglycemia produced by alkali administration (Underhill, 1916), Macleod found sugar retention by the liver of intact animals only when the perfusing fluid was alkaline.   No similar retention of sugar was observed when the dextrose solution was neutral or acid or made hypertonic by NaCl. Shortly thereafter, McDanell and Underhill (1917) fed diets containing an excess of acid and basic ash to two groups of rabbits.   They noted a larger amount of glycogen stored in the livers of the rabbits receiving the basic diet.

The first evidence that acid favored glycolysis *in vitro* was presented by Langfeldt (1921).   He showed that at a pH of 7.4 to 8.0 hydrolysis of liver glycogen by liver diastase was inconsiderable but with increasing hydrogen ion concentrations there was an increase in diastatic activity.   Hydrolysis at pH 7.0 was double that at 7.4.   His statement that the presence of adrenalin shifts the optimum to the alkaline side has recently been disputed by

Visscher (1926). This worker finds acceleration of glycolysis at increasing acidities, but does not confirm Langfeldt's observation that adrenalin prevents this acceleration.

Meyerhof and Meier (1924) found (in confirmation of work on isolated frog muscle) that in living frogs the oxygen consumed only accounts for about one-fifth of the lactate disappearing from the muscles at 20°C. This proportion, however, becomes smaller as the temperature decreases. Meyerhof and Meier present the curious fact that even though similar quantities of oxygen be measured during a part of the recovery period following stimulation of frogs of similar weight, the rate of lactic acid disappearance from muscles is 4 times greater at 20°C. than at 4½°C. Expressed differently, the ratio

$$\frac{\text{Lactic acid oxidation estimated by } O_2 \text{ consumption}}{\text{Estimated lactic acid actually disappearing}}$$

changes with the temperature in intact living frogs. This suggests that the mechanism removing lactic acid in the experiments of Meyerhof on intact animals is corroborative of the old view of von Noorden and Embden (1906), that other tissues beside the muscles producing the lactic acid are responsible for lactate utilization. This view-point is further supported by the work of Janssen and Jost (1925), Barr, Himwich, and Green (1923), and by Furusawa (1926). All of these authors present evidence which indicates that other organs besides the muscles may be involved in the removal of lactate ion, whether injected or produced by a localized group of muscles.

EXPERIMENTAL.

The animals were prepared in the same manner that has been described in the preceding papers. Low values for liver glycogen were found in dogs that had presumably not been fasting. On the other hand, dogs that had been fasted for several days frequently had much glycogen in the liver. For this reason, the fasting time of the dogs is not given in the tables, as it was impossible to lend any significance to the fasting time as known.

Ether narcosis preceding amytal was found to have no influence on the variables studied.

Glycogen was determined by Pflüger's (1910) method in duplicate or triplicate, occasionally, from different lobes only after the injection. The method of Bertrand (1920) was employed for estimation of glucose (see Evans, 1926).

Blood sugar was determined by the method of Hagedorn and Jensen (1923) and corrected for blood volume by hemoglobin estimations in a Duboscq colorimeter, and blood CO_2 as heretofore.

Blood lactate was measured as previously described (Paper I of this series) and muscle lactate was also estimated by the method of Clausen (1922); precipitation of the proteins, however, was accomplished by trichloroacetic acid when determining muscle lactate.

CO_2 was determined by the method of Van Slyke (1917).

Oxygen Consumption.—The methods and sources of error of the measurement of oxygen consumption will be discussed along with the relative experimental data.

TABLE I.

Experiment No.	Weight of dog.	Anesthesia.	Injection time.	Dose of sodium r-lactate as lactic acid.	Volume of solution.	Blood sugar before injection.	Blood sugar after injection, mg. per cent.						
							½ hr.	1 hr.	1½ hrs.	2 hrs.	2½ hrs.	3 hrs.	4 hrs.
	kg.		*min.*	*gm.*	*cc.*	*mg. per cent*							
30	8.2	Amytal.	20	10.5	150	80	62					66	73
31	7.5	"	55	20.0	300	93		89	72	69		70	67
33	9.7	"	180	30.0	600	76		65	47		38	34	
38	8.0	"	25	10.8	160	81	84		63		68		65
36	8.2	"		None.		102			108			92	109

TABLE II.

Experiment No.	Weight of dog.	Glucose injected.	Injection time.	Liver glycogen.	
				Before injection.	After injection.
	kg.	*gm.*	*hrs.*	*per cent*	*per cent*
67	5.5	30	3	0.56	1.7
72	7.5	23	2.3	0.1	0.4
68	7.0	None.		8.7	8.5

Results.

Blood Sugar.—As noted in Paper I, amytal does not influence the blood sugar, R.Q., or blood CO_2. The intact dog under amytal anesthesia behaves differently toward injections of lactate than the phlorhizinized dog and the phlorhizinized liver (Mandel and Lusk, 1906; Barrenscheen, 1914).

As Table I shows, there is no demonstrable sugar synthesis

following or during lactate administration, if the blood sugar curve be a function of the sugar of the tissues. In fact a lower blood sugar has been observed in our four experiments. The view that this drop in blood sugar is a specific lactate effect must be guarded against. The second article of this series has demonstrated the presence of an alkalosis, and the drop in blood sugar observed may very well be due to this (Underhill, 1916).

Janssen and Jost (1925) reported no change in the blood sugar of their animals following lactate administrations. They do not state, however, whether their values were corrected for blood volume. As the blood volume often diminished following the diuresis incidental to lactate injected intravenously, it is probable that the relatively constant blood sugar noted by Janssen and Jost may be due to the fact that their values are uncorrected for blood volume.

Glycogen Synthesis.—The evidence presented in the introduction leads to the assumption that, contrary to the results of the perfusion experiments of Meyerhof on frog muscles, the muscles of the intact dog do not demonstrably synthesize glycogen from lactic acid or sodium lactate. The data to be presented are concerned with glycogen formation by the liver from sodium r-lactate.

Glycogen Synthesis during Amytal Anesthesia.—That the liver still retains its ability to store glycogen during amytal anesthesia is shown by Table II. The amytal anesthesia was initiated with ether.

Glycogen Synthesis from Sodium r-Lactate.—The outstanding feature of the data presented in Table III is that, contrary to the assumptions of Elias and Schubert (1918) and Janssen and Jost (1925), *there is no glycogen synthesis by liver following lactate administration intravenously.* Actually the four experiments done with previous sampling showed a lowered liver glycogen. In Column 12, the lactic acid in the tissues was estimated by considering 70 per cent of the body weight to be in equilibrium with the blood lactate when the animal was killed. The muscle lactate, a mean of three separate samples, agrees well with the concentration found in simultaneous blood samples (see Experiments 82 and 83). So the error introduced by taking the concentration of lactate in the blood equal to that in the tissues is not too far off to give an idea of the amount of lactate utilized. The low muscle lactates also

TABLE III.

Influence of Intravenous Injection of Sodium r-Lactate on Liver Glycogen.

Experiment No. (1)	Anæsthesia. (2)	Weight of dog. (3) kg.	Dose of sodium lactate as lactic acid. (4) gm.	Volume of sodium lactate solution. (5) cc.	Dose. (6) gm. per kg.	Injection time. (7) hrs.	Length of experiment. (8) hrs.	Blood lactic acid at end of experiment. (9) mg. per cent	Total urinary excretion of lactic acid. (10) gm.	Lactic acid retained. (11) gm.	Calculated lactic acid in tissues. (12) gm.	Calculated lactic acid utilized. (13) gm.	Calculated lactic acid utilized. (13) gm. per kg. per hr.	Liver glycogen. (14) Before injection. per cent	Liver glycogen. (14) After injection. per cent	Remarks.
56	E.A.	8.0	27.4	300	3.42	2.0	2.0	240	6.7	20.7	11.8	8.9	0.56	1.0	0.2	Base excreted equivalent to 1.4 gm. lactic acid.
50	A.	5.5	15.7	330	2.85	2.6	3.1	170	3.5	12.2	5.3	6.9	0.42	Not done.	0.1	Base excreted equivalent to 4.2 gm. lactic acid.
82	E.A.	6.0	15.6	180	2.6	1.5	2.0	185	3.1	12.5	6.5	6.0	0.5	0.1	<0.1	Muscle lactic acid 190 mg. per cent at end of experiment.
59	A.	10.0	19.7	200	1.97	2.5	2.5	119	4.3	15.4	5.5	9.9	0.41	0.2	<0.1	Base excreted equivalent to 2.4 gm. lactic acid.

Exp. No.	Anesthetic														Notes
83	E.A.	9.0	15.8	155	1.75	1.0	2.6	95	4.0	11.8	4.2	7.6	0.34	1.2	0.7	Muscle lactic acid 100 mg. per cent at end of experiment.
62	"	10.0	15.4	200	1.54	1.3	1.3	Not done.	Not done.					0.9	0.3	
57	"	17.0	70.0	500	4.1	1.7	1.7	" "	" "					Not done.	<0.1	Dog died during injection. Blood lactate 543 mg. per cent.
33	A.	9.7	30.0	600	3.1	3.0	Not done.	" "	" "					" "	<0.1	

E. = ether.
A. = amytal

furnish more definite evidence that storage of lactate as such in the muscles does not take place.   (Liver lactates not reported in this communication have also been significantly low.)   Then:

(1) Total lactic acid (Column 4) — urine lactic acid (Column 10) = lactic acid retained (Column 11).
(2) Lactic acid retained (Column 11) — (0.7 × (body weight per kilo) × (gm. blood lactic acid per liter)) = lactic acid utilized in gm. — 0.3 gm. (approximate normal value) (Column 13).

The first four experiments given in Table III are *comparative* in so far as the injection time was practically as long as the experiment.   There was a constant excess of lactate ion present in the tissues.   The values for rate of utilization per kilo agree so well in the experiments on animals of such different size, and with such different doses of lactic acid, that one is inclined to accept the values found.   The mean value, about 0.5 gm. per kilo per hour, is the estimated rate at which lactate has been utilized—or with the foregoing evidence, *oxidized*.

It is not insinuated that no synthesis to carbohydrate does take place; but in view of (1) the drop in blood sugar, (2) the absence of glycogen synthesis in muscle and liver, (3) the fact that the basal oxygen consumption of the dogs of 2500 to 4500 cc. per hour easily accounts for the estimated 2.7 to 5.0 gm. per hour of utilized lactate (1 gm. of lactic acid = approximately 800 cc. of oxygen), the simplest explanation seems to be that which would fit in with other phenomena recognized to take place in the metabolism of the entire organism.   The tissues are flooded with a diffusible, utilizable substance, lactate ion.   When glucose or any easily utilizable carbohydrate is administered in the same way, the R.Q. approaches unity.   There is no reason to suppose that during the administration of lactate, oxidation does not similarly take place—particularly in the light of the data concerning glycogen synthesis presented above.   The analogous experiments on isolated tissues (Meyerhof) have been discussed in detail in the introduction.   That the isolated tissue does synthesize and the intact animal does not synthesize glycogen from added sodium lactate is a suggestion which is most compatible with the experimental evidence presented here.   The presence of other tissues and the glands of internal secretion ever present in the intact

TABLE IV.

Synthesis of Glycogen from Glucose by the Liver during Alkalosis.

Ether + amytal anesthesia.

Experiment No.	Weight of dog.	Dose, intravenous.	Injection time and duration of experiment.	NaHCO₃	Liver glycogen.		Remarks.
					Before injection.	After injection.	
	kg.		hrs.	gm. per kg. per hr.	per cent	per cent	
66	8.0	23 gm. glucose, 8 gm. NaHCO₃ in 330 cc.	2.5	0.4	3.5	3.0	Only experiment in which no rise has been observed. 2.9 gm. NaHCO₃ in urine.
74	7.0	21 gm. glucose, 7 gm. NaHCO₃ in 250 cc.	2.2	0.46	0.1	0.5	
78	5.7	32 gm. glucose, 11 gm. NaHCO₃ in 450 cc.	4.0	0.48	0.1	0.7	
79	6.5	30 gm. glucose, 10 gm. NaHCO₃ in 400 cc.	2.0	0.77	0.1	1.3	4.2 gm. NaHCO₃ in urine.
84	10	2 gm. NaHCO₃ alone before; then 40 gm. glucose, 10 gm. NaHCO₃ in 400 cc.	3.3	0.36	1.1	1.6	Blood CO₂ curve corrected for blood volume. Zero time, 47 vol. per cent; after NaHCO₃, 61 vol. per cent; at end of experiment 84 vol. per cent.
85	12	5 gm. NaHCO₃ alone before; then 60 gm. glucose, 15 gm. NaHCO₃ in 500 cc.	2.9	0.57	2.8	3.7	Blood CO₂ as above. Zero time, 46 vol. per cent; after NaHCO₃, 81 vol. per cent; at end of experiment 77 vol. per cent. 9.4 gm. NaHCO₃ in 1100 cc. urine.

animal create a system so different that the predominance of one mechanism in the first case and of another mechanism in the second, is certainly not unexpected.

One more control must be mentioned, the ability of the liver to synthesize glycogen from glucose during alkalosis.

Glycogen Synthesis from Glucose during Alkalosis.—Reference to Table IV discloses the fact that the liver in the intact animal synthesizes glycogen from glucose during an alkalosis produced by injecting $NaHCO_3$ at about the same rate that it is formed during lactate utilization as above (1 mol of lactic acid burned forms $\frac{84}{90}$ mols of $NaHCO_3$).

Oxygen Consumption.—By using the technique described above, the rôle played by the lactate ion in stimulating oxidation in the intact resting animal was relatively easily studied.   The concentration of lactate ion in the blood and tissues of the experimental animals was as high or higher than the lactate ion concentration found after most severe muscular exercise (Hill, Long, and Lupton, 1924).   The comparison of the oxygen consumption of the intact resting animal and of the exercised animal, both having approximately the same lactate ion concentration in blood and tissues, should determine the rôle played by lactate ion alone in determining the speed of tissue oxidations.

Sources of Error.—The figures given in Table V were obtained by studying animals under amytal anesthesia during intravenous injections of solutions which were hypertonic and which also were always somewhat below the temperature of the injected animal. Injections of NaCl under the same conditions either do not produce any change in oxygen consumption or produce a rise.   At all events the greatest error possible under the experimental conditions would produce an increase in metabolism.   Readings could be made to 5 cc. of oxygen.   A modified Roth (1922) apparatus was employed.

<div align="center">DISCUSSION.[1]</div>

As noted previously, the concentration of lactate ion produced in the blood and tissues by the intravenous injection of sodium lactate is as high or is higher than that found following the severest

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[1] See protocols in Table V also.

TABLE V.

Oxygen Consumption Following Intravenous Injection of Sodium r-Lactate and NaHCO₃.

Experiment No.	Weight of dog.	Injection time.	Substance administered.	Volume.	Dose.		Basal O₂.	Highest increase in metabolism observed during experiment.	Approximate time of maximum increase in metabolism after start of injection.	Remarks.
	$kg.$	$min.$		$cc.$	$gm.$	$gm.\ per\ kg.$	$cc.\ per\ min.$	$per\ cent$		
15	12.0	26	Sodium r-lactate.	150	12.0	1.0	99	+20	15 min.	
16	5.5	20	"	90	6.8	1.2	44	+34	30 "	
19	13.5	22	"	225	15.2	1.1	90	+22	40 "	
61	6.5	17	"	60	4.0	0.61	40	+17	50 "	Blood lactate 156 mg. per cent at end of injection.
62	10.0	50	"	200	1.70	1.7	70	+20	60 "	
24	7.5	30	NaHCO₃	100	5.6	0.75	51	+26	40 "	
69	6.0	140	"	300	9.0	1.5	32	+47	2 hrs.	
70	6.0	180	"	400	16.0	2.66	42	+25	Practically continuous after 1 hr.	
75	9.0	135	"	300	12.0	1.3	68	+20	Practically continuous after 45 min.	
77	9.0	100	"	240	10.0	1.1	63	+16	Practically continuous after 50 min.	

muscular exercise (see Evans (1925), "Recent Advances in Physiology"). It is well known that the resting oxygen consumption may be increased at least 10 times—1000 per cent—by severe muscular exercise. Compared to this value, the rise following slow or rapid injections of sodium lactate *produces a comparatively negligible rise in the oxygen consumption.* That this specific dynamic effect of lactate ion is relatively negligible in these experiments is further emphasized by the fact that injections of sodium bicarbonate, in amounts equivalent to that produced during lactate oxidation, lead to a rise in metabolism of the same order of magnitude. Since the specific dynamic action of substances is believed to be additive, this would lead to the inference that the specific dynamic action of lactate ion is even less than observed.

These experiments, then, do not bear out the view that lactate ion is alone responsible for the increased metabolism observed following muscular exercise. The reason for increased oxidation in muscular exercise apparently must be sought for in other sources, such as cardiovascular and other similar mechanical as well as chemical changes.

SUMMARY.

1. Isolated mammalian tissues (liver, muscle) synthesize carbohydrate (glycogen) in an alkaline medium (Meyerhof) from lactate.

2. The muscles of the intact dog do not synthesize carbohydrate from perfused lactic acid (Elias and Schubert) or from sodium d- and r-lactate (Janssen and Jost) (confirmed by the authors).

. 3. During or following intravenous injection of sodium r-lactate producing as high, or higher, values of lactate in the blood and muscles occurring in most severe muscular exercise (*a*) the blood sugar drops slightly, (*b*) the glycogen in the liver diminishes, (*c*) the increase in oxygen consumption is negligible compared to the rise following severe muscular exercise, (*d*) it is estimated that the intact anesthetized dog utilizes about 0.5 gm. per kilo per hour of lactic acid. This amount is approximately balanced by the basal oxygen consumption.

4. Slightly hypertonic $NaHCO_3$ produces a well marked rise in metabolism, thus diminishing the lactate effect observed.

5. Under amytal anesthesia, the liver retains its ability to syn-

thesize glycogen from glucose even though a marked alkalosis is present.

CONCLUSIONS.

1. The intact anesthetized (amytal) dog does not demonstrably synthesize glucose or glycogen (liver) from sodium r-lactate.

2. The lactate ion *per se* plays a negligible rôle in the stimulation of tissue oxidations of the intact dog under the same conditions.

Dr. Manfred S. Guttmacher has made most of the measurements of oxygen consumption. We are indebted to Professor A. V. Hill for his criticisms and encouragement throughout the course of this work.

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THE RELATION OF THE RATE OF GROWTH
TO DIET. II.*

By LAFAYETTE B. MENDEL AND HELEN C. CANNON.

*(From the Laboratory of the Connecticut Agricultural Experiment Station
and the Laboratory of Physiological Chemistry, Yale University,
New Haven.)*

. In 1926 Osborne and Mendel (1) published a report of some of
their experiences in securing in male rats, kept under laboratory
conditions, more rapid growth than has hitherto been recorded
for this species in the literature with which we are familiar. The
conclusion was reached that the favorable outcome of our experi-
ments was not due to selection or marked changes produced in
our stock through breeding, but was primarily the result of a more
appropriate diet than had hitherto been employed regularly in
the experimental feeding of rats. This implies that, owing to the
shortcomings of the rations used, the inherent capacity of the rat
to grow has in the past rarely been given full play in the laboratory.
Consequently the published records and compilations of "norms"
fail to furnish an adequate idea of the rate of growth of which the
rat is capable.

In the most familiar records of growth, such as are presented in
Donaldson's "The Rat," the average time required for the change
in body weight in the male from 60 to 200 gm. is 79 days; from 60
to 275 gm., it is 244 days. The corresponding figure for the female
growing from 60 to 200 gm. is 129 days. The *average* daily
gains calculated from these figures are as follows:

*Average Daily Gain in Body Weight in Growing Rats. Donaldson's
Standard.*

	Males.	Females.
	gm.	gm.
From 60 to 200 gm	1.77	1.09
" 60 " 275 "	0.88	

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* The expenses of this investigation were shared by the Connecticut
Agricultural Experiment Station and the Carnegie Institution of Wash-
ington, D. C.

The twenty *best* records of average daily gains in weight as observed in our experiments are at present as shown in Table I.

The records of rapid growth presented in an earlier paper referred to male rats.   The data just cited indicate that through suitable dietary provision acceleration in weight gain can likewise be promoted in the females.   Except for a brief early period the

TABLE I.

Record Rates of Growth of Rats.

Males.			Females.		
Rat No.	Duration of growth from 60 to 300 gm.	Average gain per day.	Rat No.	Duration of growth from 60 to 200 gm.	Average gain per day.
	days	*gm.*		*days*	*gm.*
B3601	39	6.1	B3514	35	4.0
B3414	39	6.1	B4345	37	3.8
B3441	40	6.0	B3468	37	3.8
B3380	44	5.5	B3479	38	3.7
B3421	44	5.5	B4289	39	3.6
B2161	44	5.5	B4210	39	3.6
B2132	45	5.3	B3917	39	3.6
B2164	45	5.3	B4365	41	3.4
B1133	45	5.3	B3472	41	3.4
B2705	46	5.2	B4758	42	3.3
B3212	47	5.1	B4363	42	3.3
B3218	47	5.1	B3473	45	3.1
B3425	48	5.0	B4209	45	3.1
B2328	48	5.0	B3437	45	3.1
B2329	48	5.0	B4341	46	3.0
B581	49	4.9	B3440	46	3.0
B3548	49	4.9	B3481	47	3.0
B4776	49	4.9	B4212	50	2.8
B3506	50	4.8	B3422	50	2.8
B2258	50	4.8	B4370	54	2.6

females tend under any condition to grow at a slower rate than the males similarly maintained; and in general they attain a smaller adult size than the latter.   This difference between the sexes remains apparent when the growth rate is augmented to the optimum extent; but even during the period of most rapid augmentation the maximum daily gain in weight of the females is decidedly less than that of the males.   However, the hitherto

published "average" rates of growth of *both* sexes of the albino rat are decidedly below the readily attainable optimum. The sustained excellent gains noted in our records above are in no respect freak results. Daily gains of more than 4 gm. per day, in contrast to the conventional 2 gm. or less, have been observed in many animals during the period of most active growth, in experiments too numerous to record here.

Notably rapid gains in weight have hitherto been observed under conditions in which realimentation occurs after marked loss of weight for one cause or another; likewise when growth is resumed after a period of cessation of growth with stationary body weight at a state of development when larger size is still to be expected. In studying the curves of growth of a considerable number of albino rats in which, for a diversity of reasons, growth had been inhibited for varying periods, we were impressed more than 10 years ago by the unexpectedly accelerated rate at which the increment of body weight may be resumed when the conditions become favorable. Illustrative charts had already been published (2) showing "curves of repair" after a considerable decline in body weight due to feeding with a defective diet. They were stated (3) to indicate that:

"Under suitable dietary conditions lost weight may be regained far more rapidly than during normal growth through the same range of body weight. It was pointed out in our earlier publication that the chemical or metabolic processes of repair are probably by no means identical with growth. They may not involve the destruction and resynthesis of an entire protein molecule or of the entire protoplasmic cell structure. It is, furthermore, a familiar fact that repair or recuperation can take place at all ages, even after the completion of ordinary growth in the individual."

At the time when these experiments were concluded we assumed from comparisons with the so called normal growth records then available, that "resumed growth" not only may occur at a rate normal for the size of the animal at the time, but frequently exceeds the usual progress (4). It was noted that the size or age at which the inhibition of growth is effected does not alter the capacity to resume growth. Even when the suppression of growth is attempted for very long periods at a very small size (body weight), the restoration may be adequate when a suitable diet is furnished. In the light of our recent experiences it became of interest to com-

pare the actual rates of gain in these earlier "recovery" experiments with the best gain records now available.   Table II summarizes some of the observations secured with animals of different sizes.

All these results were secured some time ago, before we were familiar with the dietary conditions that favor the rapid growth

TABLE II.

Average Gains of Weight per Day during Periods of Most Rapid Resumption of Growth Following Suppression of Growth Due to Various Dietary Causes.

	Males.					Females.			
Rat No.	Age when re-sumption began.	Body weight when re-sumption began.	Duration of rapid gain.	Average gain per day.	Rat No.	Age when re-sumption began.	Body weight when re-sumption began.	Duration of rapid gain.	Average gain per day.
	days	*gm.*	*days*	*gm.*		*days*	*gm.*	*days*	*gm.*
4623	58	86	14	7.6	7854	79	44	7	7.0
4769	129	157	10	7.2	B794	54	45	14	7.0
2911	187	160	10	6.9	4031	528	160	11	6.6
4839	92	84	7	6.9	B3483	59	43	21	6.3
8416	65	105	11	6.8	5466	129	122	10	5.7
5817	107	155	7	6.8	4876	110	131	10	5.7
7094	376	244	8	6.7	2559	132	102	7	5.0
4690	112	125	10	6.6	4877	110	143	7	5.0
4881	81	97	7	6.5	B2856	214	145	18	4.9
4880	88	127	7	6.4	4246	101	66	7	4.9
4857	107	178	10	6.4	5440	64	50	10	4.8
4855	79	88	7	6.4	B816	50	40	10	4.8
B648	88	160	14	6.2	B788	51	53	14	4.5
5350	109	138	7	6.1	6446	69	49	10	4.4
5723	114	195	9	6.0	B2756	56	40	14	4.2
3188	93	138	7	6.0	4209	148	131	7	4.0
2733	371	153	14	5.9	4661	59	76	21	4.0
4400	240	215	7	5.9	B2906	81	70	11	4.0

under discussion earlier in this paper.   The realimentation rations formerly used were varied in character.   We have recently conducted new experiments involving repair or renewal of growth after suppression thereof and supplying the "recovery ration" in the form of foods recently demonstrated to promote gains in

adolescent animals at a rapid rate. The new data do not include any results, however, that surpass those recorded above, and therefore they need not be reproduced here.

Inasmuch as the capacity to grow inevitably decreases with increasing size in animals, it is futile to expect that equally rapid gains can be made at all stages of development. The younger period of course represents the most promising time for securing outstanding gains. For this reason the present studies either have been referred to periods of growth beginning at a normally attained body weight of about 60 gm., or the initial sizes of the rats are designated in the protocols. By comparison of the statistics in Tables I and II, it becomes apparent that *with suitable food* the rapid gains of "recovery periods"—which are notable for the efficacy of securing gain in weight—rarely surpass the increments made under the best conditions of uninterrupted growth. This means, at a body size of 100 to 200 gm., an average gain of at least 5 gm. per day. In view of such results one may raise the question whether it is necessary to postulate any extraordinary capacity for gain incident to resumption of growth after it has been suppressed. Perhaps the increment of weight under such conditions is merely illustrative of what can take place at any early period of growth, provided the proper food factors are supplied.

In his interesting investigations of the function of the anterior hypophysis Evans (5) has directed attention to the remarkable potency of the gland in relation to growth. He concluded that "the anterior hypophysis is indispensable for growth to adult stature, a lessened amount of its hormone being the direct cause of an important group of, if not all, endocrine dystrophies, and an increased amount of the hormone the direct cause of overgrowth." The latter conclusion is based in part on experiments in which rats were treated with suitable hypophyseal substance. Evans stated that:

"*Rats treated parenterally with fresh anterior hypophyseal fluid invariably possess a greatly altered third, or post puberal growth curve.* The rate of growth does not at 'adulthood' undergo the sudden decline characteristic for normal animals and indeed many of our rats did not sensibly alter their growth at this epoch. It was in this epoch consequently, in the epoch which begins somewhere between the ninetieth and one hundred and

fiftieth day of life, that a very great disparity began to show in the growth of treated and untreated rats. . . . The anterior rats, having to contend daily against the disadvantages of this treatment, were visibly becoming gigantic animals. . . . The hypophysis giants were, in late stages, twice as heavy as the largest individuals known to us from our own and the published records for this animal species. Studies of these animals showed that they were not merely fat animals but that a true overgrowth

TABLE III.

Average Gains of Weight per Day After Injections of Hypophysis Extracts in Rats. (Selected from Evans' Protocols.)

Rat No.	Females.			
	Growth:			Average gain per day.
	From	To	In	
	gm.	*gm.*	*days*	*gm.*
GH5525	61	208	30	4.9
W5509	56	221	35	4.7
W5406	55	215	35	4.6
W5523	65	203	30	4.6
BH5487	60	198	30	4.6
W5492	65	197	30	4.4
BH5503	61	195	30	4.4
B5529	63	218	35	4.4
W5542	61	214	35	4.4
GH5493	66	194	30	4.3
B5401	59	209	35	4.3
B5498	68	192	30	4.1
GH5513	69	208	35	4.0
B5411	66	204	35	3.9
W5521	64	201	35	3.9
GH5510	64	199	35	3.9
W5484	65	200	35	3.9
B5505	66	201	35	3.9
BH5534	60	213	40	3.8
W5500	67	197	35	3.7

was participated in by the skeleton and by most of the viscera, especially the heart and lungs, liver, kidney and alimentary tract, but not by the reproductive tract, uterus and oviduct remaining infantile.''

We have ventured to ascertain from Evans' published records whether these uniquely treated animals gained at a more rapid rate than our vigorously growing rats developing under what may

be regarded as essentially normal conditions, in which advantageous feeding provisions alone were made. Table III includes selected figures from Evans' protocols for such rats—data for females only being recorded—and such sizes and periods as will permit comparison directly with our own finding on uninjected but well fed animals. The gains per day of the rats receiving hypophysis parenterally during the growth period represented by a size of about 60 to 200 gm. of body weight include one maximal record of 4.9 gm. along with several above 4.0 gm. per day. Comparison with our records, for females, in Table I shows that the administration of the hypophysis extract, during the period of adolescence here represented at least, has not augmented the rate of growth very greatly beyond what has been accomplished by diet alone in our tests. These comments obviously do not refer to the later periods of life in which the females developed into what Evans designates as "hyperpituitary giants." They raise the question, however, whether the treatment with pituitary does not serve primarily to modify adult development rather than the growth of the more typically adolescent period.

It might be expected that the apparent precocity exhibited in growth in our newer feeding experiments would result in earlier sexual maturity. This raises the question whether the development of the various organs keeps proper pace with the rapid growth of the body as a whole. Such breeding tests as we have undertaken have not indicated any tendency to produce young at the early age that might be expected from the *size* of the rapidly growing rats. However, the records of the age of pubescence are still too variable to permit any final statement. In our experiments males and females were kept on a diet that had demonstrated its efficiency in promoting rapid gains and that contained a liberal inclusion of wheat germ to provide vitamine E. They were placed together in cages during a part of each day at a period considerably before sexual maturity could have been attained. The age at which actual successful mating occurred is estimated for the date 22 days before the young were born. A few actual records are given in Table IV.

TABLE IV.

Sexual Maturity of Rapidly Growing Female Rats.

Female.	First placed with male at:		Sexual maturity indicated by beginning of pregnancy at:		Average daily gain during 40 days preceding pregnancy.	No. of young born.
	Age.	Body weight.	Age.	Body weight.		
	days	*gm.*	*days*	*gm.*	*gm.*	
B4634	53	139	58	135	2.3	6
B4636	49	137	64	162	2.8	3
B4629	46	120	65	170	3.1	6
B4617	51	135	70	169	2.7	7
B4618	51	128	90	214	2.1	6
B4630	44	113	96	240	2.4	6

Referring to female rats Evans and Bishop (6) have reported that "the approximate age of 60 to 70 days for maturity as given by Donaldson . . . . differs somewhat from what may be attained by animals on a superior nutrition, in which a relative precocity results." In a record of several hundred females the age of sexual maturity ranged from 30 to 94 days. Slonaker and Card (7) state that sexual maturity occurs between the ages of 60 and 90 days. Kirkham and Burr (8) reported successful mating at the age of 55 days.

SUMMARY.

Additional records are presented of the growth of both male and female albino rats showing rates of gain notably greater than those usually regarded as "normal" for this species. Whereas the figures most widely referred to indicate average daily gains, during the period of increment from 60 to 200 gm. of body weight, of 1.77 gm. for the males and 1.09 for the females, our newer experiments frequently showed gains above 5.0 and 3.0 gm. respectively for comparable stages of development. These better gains are clearly attributable to improved dietary conditions and may be regarded as more nearly optimum growth for rats.

Comparisons of the well known rapid rates of vigorous gain during periods of recovery from loss of weight or other interruption of growth with the new records of uninterrupted gain on a suitable regimen indicate that the best growth under the latter

conditions is rarely much exceeded by the best increments during "recovery." In other words, the best "curves of repair" scarcely surpass the most satisfactory "curves of normal growth," when the diet is appropriate. The possible biological significance of this is discussed.

Comparisons are recorded between the rate of growth of Evans' "hyperpituitary giant" female rats during adolescence and our most successfully nourished females. The effect of the rapid growth rate on the development of sexual maturity has been considered. Tendency to produce young at exceptionally early ages has not been observed.

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THE MINERAL CONTENT OF HUMAN SKIN.

BY HERMAN BROWN.

(From the Department of Chemistry, Research Institute of Cutaneous Medicine, Philadelphia.)

A knowledge of the chemical composition of the human skin and a demonstration of any changes which may occur in its chemistry during growth is a prerequisite for the study of the various diseases peculiar to that organ. Evidence of the rôle played by the mineral constituents of the skin in its normal metabolism as well as in its pathological processes has gradually been accumulating and the literature bearing on this subject has been reviewed in previous communications (1, 2).

The object of the present paper is the presentation and discussion of data obtained in the analyses for the mineral content of over forty samples of human skin.

Procedure.

Determinations were made for moisture, ash, calcium, magnesium, sodium, potassium, silicon, and phosphorus. The methods used were, in general, similar to those previously described. However, owing to the greater number of determinations made on any one sample of skin, certain modifications were introduced which will be described here.

The strip of skin, obtained at necropsy through the kind cooperation of Dr. Eli R. Saleeby, freed from hair, superficial fat, and subcutaneous tissue by scraping with a knife, was weighed and dried to constant weight at 105–106°C. The loss in weight was taken as the moisture content of the skin. The skin was ashed according to the method of Stolte (3) in a tared crucible, the ashing being continued until no carbon particles were visible. The crucible was weighed to obtain the amount of ash. The ash was then treated in the conventional manner for the gravimetric

determination of silica, the silicon being calculated from the loss in weight after treatment with hydrofluoric and sulfuric acids.

A few determinations of silica were made by Issacs' (4) colorimetric method with a separate piece of skin. Bertrand's (5) criticism of the interfering effects of phosphorus caused some concern since skin contains relatively large amounts of phosphorus. However, the results were essentially of the same order as those obtained gravimetrically, and Foulger's (6) recent exhaustive study of Issacs' method indicates that the colorimetric procedure is sound.

The hydrochloric acid-soluble portion of the ash was made up to a definite volume and aliquot portions taken for analysis. One portion was used for the calcium and magnesium determination and a second portion for the sodium and potassium determinations. The methods for these were given in a previous communication (2). Phosphorus was determined in a third portion by double precipitation as the phosphomolybdate according to Woy's technique and subsequent precipitation with magnesia mixture (7).

<div align="center">DISCUSSION.</div>

The moisture, sodium, potassium, and phosphorus content of over forty samples of skin are listed in Table I and the variations of the ash, calcium, magnesium, and silicon with age are plotted (Fig. 1). Analytical errors plus the individual variations which exist for any one constituent make it impossible to place the other values on a curve.

The curve for the ash requires some comment. Moulton (8) has summarized the results of various investigators on the ash content of the entire animal and has shown that for the whole body there is a continual increase in the ash up to a definite age, characteristic for each species, at which point the ash content becomes practically constant. This point he has designated as the age of "chemical maturity" for that particular species. However, a consideration of the ash of the entire body means essentially merely a consideration of the ash of the bones. Thus any variation in the curve of the ash of individual organs is obscured owing to the preponderating influence of the ash of the bones. This is indicated in the results obtained for the skin, for while the skin of the fetus exhibits mineral-storing capacity equal in rate to the rest

TABLE I.
Mineral Content of Human Skin.

Sample No.	Name.	Race.	Sex.	Age.	Moisture.	Mg. per 100 gm. original skin.		
						Na	K	P
					per cent			
187	M. W.	Black.	F.	65 yrs.	61.2			
185	S. R.	"	"	38 "	60.7			
186	C. S.	White.	M.	67 "	59.8		113	66
207	A.M.	"	F.	75 "	67.3		68	58
208	L. J.	"	"	51 "	58.7		53	45
212		Black.	"	44 "	66.5		90	69
211		White.	M.	38 "	57.4		104	71
213	M. H.	Black.	F.	28 "	56.4		98	65
238	L. C.	"	M.	24 "	62.3		105	43
240	M. B.	"	F.	33 "	66.1		116	47
252	D.M.	"	M.	27 "	63.4		112	54
253	W. C.	White.	"	78 "	65.5		70	46
254	R. H.	"	F.	62 "	67.9		64	41
259	J. N.	"	M.	2 days.	68.6	118	161	72
284	F. S.	"	"	82 yrs.	66.2	160	93	55
287	L.D.	Black.	F.	28 "	69.8	119	85	60
289	A. J.	"	"	35 "	71.1	181	66	41
290	V. J.	"	M.	53 "	58.2	184	58	54
296	E. C.	"	F.	2 "	66.3			
292	P. S.	"	"	20 "	58.8	132	99	59
304	S. W.	"	M.	32 "	63.4	161	88	65
310	A. H.	White.	"	46 "	63.1	138	111	
317	E.	Black.	F.	40 "	62.4	151	126	
323	A. U.	White.	M.	63 "	64.6	177	92	
327		Black.	"	8 "	64.5	158	64	
337	B. T.	White.	F.	60 "	62.1	182	78	
338	L. H.	Black.	M.	44 "	62.5	159	94	
355	D. M.	White.	"	71 "	65.7	162	82	
359	A. O.	"	F.	73 "	68.3	147	95	
368	W. C.	"	M.	60 "	57.5	176	93	
369	W. J.	Black.	"	38 "	61.3	121	116	
373		White.		7 mos. fetus.	72.7			
394	F. G.	Black.	M.	35 yrs.	59.2	183	78	75
395	C. W.	White.	"	66 "	64.5	167	114	85
396	R. D.	"	"	25 "	59.0	161	58	62
410	A. R.	Black.	"	14 "	66.5	159	65	
535	B. J.	"	"	24 "	64.0	151	82	69
J 1		White.		5 mos. fetus.	63.1	179	86	
J 2		Black.		8 " "	62.9	159	78	
J 3		"		6 " "	66.7	138	106	
J 4		White.	F.	19 yrs.	57.5	148	65	68
648		"	M.	10 mos.	63.8	188	134	82
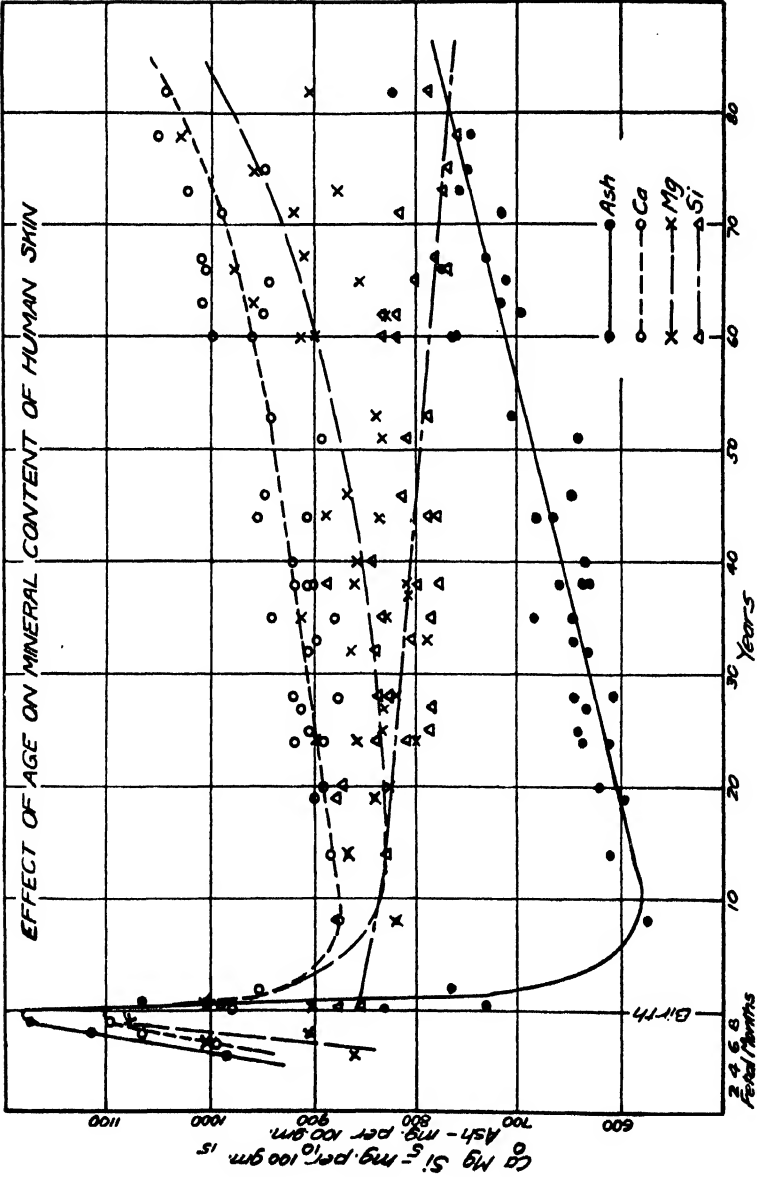
FIG. 1.

of the body, as summarized by Moulton, yet at birth there begins a sharp decline in the ash content which continues until about the end of the first age decade when the curve again begins to rise. This rise is steady and the curve shows no tendency to become asymptotic at any age. There is thus no evidence of a chemical maturity as far as the ash of the skin is concerned.

Similar considerations apply to the calcium content of the skin, since the curve of the calcium follows the same general trend as that of the ash. The same may be said of magnesium which parallels rather closely the calcium curve. It is interesting to note, however, that magnesium changes somewhat more abruptly than calcium in the skin. That is, when calcium is high, magnesium has almost the same numerical value (it is even higher in one case) while low calcium figures are accompanied by much lower figures for magnesium.

It is to be regretted that the figures for the alkalies do not exhibit sufficient regularity to be plotted. The reasons for this have been pointed out. However, since the ash content represents the summation of the fixed base and fixed acid, it might be assumed that sodium and potassium likewise change in the same manner as the ash with age.

Attention may here be called to the curve for silicon. Due principally to the work of Luithlen (9) it has been assumed that there is a marked decrease in this element in the skin of old persons and on this basis various compounds of silicon have been proposed and used, mainly in Germany, for the treatment of the degenerative changes in the skin characteristic of old age. It would appear from the curve, that, though the figures for silicon are quite variable, nevertheless there seems to be a definite trend to lower silicon values in older persons. This decline in the curve is not marked, however, and it is quite possible that any favorable clinical results reported by dermatologists due to silica therapy, may be ascribed to other factors.

There is, therefore, a constantly changing mineral content of the skin, and the assumption of a definite age of chemical maturity for the skin cell is untenable.

On the whole, the significance of these variations in the mineral content of the skin is not clear. The results are, however, interesting from a dermatological point of view. For while it has been

shown by Luithlen (10–12), Klauder and Brown (13), and others that for animal skin certain relations exist between the irritability of the skin and its mineral content, no such assumption can be made relative to human skin from the foregoing data.   Abnormalities of the skin are, in general, not confined to any one age decade and it will require intensive study of the clinical picture together with the collection of further data before any correlation can be attempted between the mineral content and irritability of human skin.   The work is being continued with this object in view and it is hoped that a further report will be made in the near future.

SUMMARY.

Data are presented for the moisture, ash, calcium, magnesium, sodium, potassium, phosphorus, and silicon content of human skin.

The effect of age upon these constituents is indicated and the relation of the mineral content of the skin to its irritability is discussed.

It is shown that most of these elements change continuously with age, indicating that there is no age of chemical maturity for the skin.

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NITROCELLULOSE MEMBRANES OF GRADED PERMEABILITY.

BY H. F. PIERCE.

(From the Department of Physiology, Columbia University, New York.)

Many investigators who have attempted to use nitrocellulose membranes in their work have found the preparation of the membranes a problem in itself, often more time-consuming than the projected investigation. The lack of precision and the number of uncontrolled conditions in the usual methods of membrane preparation led the author to endeavor to standardize the factors entering into the process, and to simplify it as much as was consistent with uniformity of results.

Nitrocellulose membranes have been employed for the study of diffusion phenomena since the time of Fick (1855) (1), who introduced them as a substitute for the vegetable and animal dialytic septa of Graham (2). He recognized their advantages, but had considerable difficulty in attaching them to suitable holders and finally abandoned their use. A few years later Schumacher (3) devised the closed nitrocellulose dialyzing sac and carried out a variety of experiments, which, however, were not numerous or systematic enough to be of importance. Baranetzky (1872) (4) made flat membranes with collodion and filter paper built up in cells on a glass plate, and noted that in order to be permeable they must be placed in water before the solvent had completely evaporated. Malfitano (5) observed that nitrocellulose sacs, made by the method of Schumacher, became impermeable when allowed to dry completely in air, and also when heated in water. He observed that collodions rich in alcohol yielded the most permeable membranes. Bigelow and Gemberling (6) found marked differences in the permeability of membranes made from different samples of nitrocellulose.

Bechhold (7) was the first to devise a practical method of preparing a series of membranes of graded permeability. He discovered that the permeability of a membrane depends upon the concentration of the colloid in the solution from which it is made. Filter papers were impregnated with glacial acetic acid solutions of nitrocellulose of various strengths, drained, and immersed in water to gel the collodion and wash the film free from

acid. Strong solutions of nitrocellulose produced less permeable membranes than those of lower concentration.

Schoep (8) prepared graded membranes from solutions of nitrocellulose in ordinary alcohol and ether to which were added different percentages of glycerol to increase and vary the permeability of the membranes, and castor oil to make the film more flexible and decrease its fragility.

Walpole (9) made membranes by pouring a measured volume of alcohol-ether collodion on a levelled glass plate of known area, allowing the collodion to dry for a definite period of time, and immersing the plate in water. After immersion the membrane was stripped from the plate and stored in water. Grading was accomplished by varying the drying time, the permeability diminishing as the drying time was increased. Permeability was defined in terms of "wetness," or the ratio of water to nitrocellulose in the membrane.

The admirable and painstaking investigations of Brown (10) produced an excellent method of preparing graded membranes of fair permeability. Dry nitrocellulose films of known weight per unit area were immersed in swelling mixtures of water and alcohol for 24 hours, and then transferred to water. The degree of swelling being a function of the alcohol content of the mixture, the permeability of the membrane could be specified in terms of the alcohol percentage of the swelling solution.

Graded membranes of high permeability have been made by Eggerth (11), by varying the proportions of absolute alcohol and anhydrous ether in the collodion employed, while holding the drying time approximately constant. The higher the alcohol to ether ratio, the more permeable is the resulting membrane. Permeability is expressed in terms of the weight per cent of alcohol in the solvent.

The method of Nelson and Morgan (12) yields reproducible membranes of the highest permeability. A definite amount of anhydrous alcohol-ether collodion is poured on a levelled glass plate of known area and allowed to evaporate to a weight which has been found by previous trials to yield suitable membranes, and the film then immersed in water. This method offers a fairly precise means of grading. Instead of plunging the partly dried films in water directly, they are carried through a series of alcohol-water mixtures ranging from 90 to 10 per cent alcohol, thus gradually replacing the solvent with water and achieving greater permeability of the membranes. Permeability is defined in terms of the ratio of water to nitrocellulose in the finished membrane. These authors note that small percentages of water in the collodion, or introduced into the drying films, greatly increase the permeability of the membranes but make them very fragile.

Asheshov (13) prepared membranes from nitrocellulose dissolved in a mixture of ordinary alcohol and ether, grading them by adding to the collodion various quantities of acetone to increase, and of amyl alcohol to decrease their permeability. He states that the drying time is an important factor, and that collodion containing more than 35 per cent of the 95 per cent alcohol produces membranes too fragile for use.

From this short résumé of the outstanding methods of preparing nitrocellulose membranes one may derive certain important principles. The permeability of such membranes is influenced by: (a) The kind of nitrocellulose employed (Bigelow and Gemberling (6)). (b) The nature of the solvent, and, when it is a mixture, the relative proportions of its constituent substances (Malfitano (5), Eggerth (11), Nelson and Morgan (12)). (c) The time of drying (Walpole (9)), the amount of swelling of dry films (Brown (10)), and the concentration of nitrocellulose in the collodion used (Bechhold (7)); hence the ratio of solvent or swelling agent to nitrocellulose in the membrane at the time of its immersion in water. (d) The addition of such non-swelling and non-solvent substances as glycerol and water to the collodion from which the membranes are made (Schoep (8), Nelson and Morgan (12), Asheshov (13)). It is to be noted that these substances also make the membranes fragile. These factors will be considered in their proper order.

Nitrocellulose is a generic term for the various nitration products of cellulose. It is usually prepared by treating cotton lint with a mixture of strong nitric and sulfuric acids, the degree of nitration depending on the time and temperature of the reaction and the equilibrium of the products with the spent acid at the end of the run. The nitration products may contain from about 7 to nearly 14 per cent of nitrogen. There is no relation between nitrogen content and solubility (14). Certain kinds of nitrocellulose are soluble in 95 per cent alcohol, others in absolute alcohol, and still others in esters, ketones, and mixtures of alcohol and ether. Cellulose nitrates containing less than 9 per cent or more than 13 per cent of nitrogen are practically insoluble in organic solvents. The army propellant usually referred to as guncotton has a nitrogen content of 13.4 per cent. There is no relationship between the nitrogen contents of these cellulose esters and the viscosities of their solutions (see Table I).

Duclaux and Wollman (15) fractionally precipitated nitrocellulose from its acetone solution by treatment with acetone-water mixtures and found that the fractions, although of identical nitrogen content, gave solutions having quite different viscosities. The same sample of nitrocellulose will yield solutions of different viscosities in different solvents. This does not necessarily indi-

cate a difference in the molecular structure of the ester particles, but in the degree to which they are surrounded and swollen by the solvent molecules (16).

Collodion is defined as a solution of nitrocellulose in a mixture of ether and ethyl alcohol. The word is used in a broader sense to designate solutions of nitrocellulose in other solvents, in which case the name of the solvent is joined to it; e.g., "glacial acetic acid collodion," "ethyl acetate collodion." The viscosity of a collodion is affected by age, light, heat, and violent agitation, the effects being due, in all probability, to a change in the state of aggregation of the molecules of solute and solvent.

Since the formulæ and structure of cellulose and its esters are still debated, there is no cellulose nitrate which is a known chemical entity. Any nitrocellulose that we may select is a mixture of several, or many, cellulose nitrates. Hence in order to have uniform material for any series of membrane experiments, it is ad-

TABLE I.

Sample No.............	1	2	3	4	5	6	7	8	9
Nitrogen, *per cent*..	11.99	11.90	12.22	12.10	11.85	12.35	11.68	11.75	12.01
Viscosity, *centipoises*........	14,028	4250	5850	11,594	8704	6460	9078	6120	11,254

visable to secure at the outset an ample quantity of suitable nitrocellulose, to mix it thoroughly, and to store it in the dark and cold.

The nature of the solvent is an important factor in the production of sound and uniform membranes. A solvent which evaporates rapidly may so reduce the temperature of the collodion that water is condensed from the atmosphere, precipitating the nitrocellulose from its solution and producing the phenomenon technically known as "blushing" or whitening of the film. The criterion of suitability of a solvent is not the boiling point but rather the vapor pressure curve. For example, ethyl acetate and absolute alcohol have approximately the same boiling point, but ethyl acetate evaporates nearly twice as fast as alcohol at ordinary temperatures (17). If evaporation is too rapid no film is formed, the nitrocellulose being deposited in an amorphous state (18). For our purpose, the solvent should evaporate slowly enough so that blushing is prevented, and should produce a smooth and uni-

form film. The fraction remaining in the film at the time of its immersion in water should be freely soluble in water so that it may be easily and quickly washed out. The solvent should not contain any substance like water or glycerol which tends to make the membrane fragile. A mixture of absolute alcohol and anhydrous ether best fulfils these conditions. An ester-soluble nitrocellulose is insoluble in ether, is swollen by alcohol, and is soluble in a mixture of the two. When collodion made with these ingredients is poured upon a glass plate and allowed to dry, the ether evaporates much more rapidly than the alcohol, and when the ether-alcohol ratio has fallen sufficiently, a gel forms. Very little ether remains in the gel, and when the plate is immersed in water, the alcohol is quickly replaced by water and a permeable membrane is produced.

The ratio of solvent or swelling agent to nitrocellulose in the film at the moment of its immersion in water is, for a given solvent and nitrocellulose, the prime factor determining the permeability of the membrane. With Bechhold's technique, filter papers of uniform thickness and texture are dipped in a glacial acetic acid solution of nitrocellulose, drained, and transferred to water. The collodion is held in the pores of the filter paper and is gelled by the action of the water which replaces the acetic acid. With a concentrated nitrocellulose solution there will be relatively less acid to replace and a more dense and impermeable membrane will be formed. In Walpole's method the solvent-nitrocellulose ratio of the film is determined by the length of time of drying; in other words, by the amount of solvent evaporated from the film before immersion. In Brown's process a dry nitrocellulose film is allowed to take up alcohol by treatment with an alcohol-water mixture. Nitrocellulose is insoluble in water; alcohol swells nitrocellulose; water restrains the swelling process. Therefore the concentration of alcohol in the swelling mixture determines the degree of swelling of the film, and hence the solvent-nitrocellulose ratio at the moment of its immersion in water. Whether one employs the method of Bechhold, the method of Walpole, or the method of Brown, the principle involved is the same; namely, control of the solvent-nitrocellulose ratio of the gel at the time of immersion in water.

Collodion to which such non-solvent substances as water or glycerol have been added yields membranes which are very permeable, but which have little tensile strength and are difficult to

remove intact from the plates on which they are made. A possible reason for this behavior suggests itself. If enough water or glycerol is added to a dilute collodion, the nitrocellulose separates out in flocks. Conceivably something of the same nature occurs in the film made from a water- or glycerol-containing collodion. In the original collodion there is enough solvent to keep both nitrocellulose and water or glycerol in solution, but as the solvent evaporates and the ratio between the solvent and the water or glycerol decreases, the nitrocellulose is partially precipitated. The more water or glycerol in the collodion, the less continuous is the nitrocellulose meshwork of the gel and the more permeable and fragile is the resulting membrane. Schoep added castor oil to his glycerol-containing collodion to strengthen the membrane. Castor oil is a swelling agent for nitrocellulose, non-volatile and insoluble in water, hence it remains in the membrane after water immersion and increases the continuity of the nitrocellulose meshwork.

The present research was undertaken after the author had attempted to make, by existing methods, membranes which could be employed satisfactorily in his physiological investigations. Numerous preliminary experiments indicated that attention should be directed to the following points: (1) the collodion; (2) the temperature of the drying film; (3) the volume, temperature, and moisture content of the air passing over the drying film; (4) the drying time.

The nitrocellulose chosen was du Pont's parlodion, not on account of any particular superiority, but because it is readily available to most of the biologists and chemists of this country. The material is in shreds and is packed in water. The shreds were drained, washed with distilled water, dried in air for 48 hours, and dried in a sodium hydroxide desiccator for 3 days.

Squibb's anesthetic ether was used in these experiments. It contains over 97 per cent of ethyl oxide and about 0.8 per cent of water, the remainder being ethyl alcohol.[1] The absolute alcohol was the chemical reagent, supplied by the United States Industrial Alcohol Company.

The alcohol and ether were mixed in the proportion of 75 per

[1] These figures are based on a large number of analyses made by an independent firm, and privately communicated to the author.

cent to 25 per cent by volume, and in each 100 cc. of this solvent was dissolved 1 gm. of the dried nitrocellulose.

To control the condition of the air and the temperature of the drying film, certain apparatus was assembled, which is shown diagrammatically in Fig. 1. The rate of flow of the air, taken from the compressed air mains of the laboratory, is controlled by an aircock and needle valve. The air is bubbled through sodium chloride brine kept at a temperature of approximately $-5°C$. by a small electric refrigerating apparatus (19), and is thereby given a constant moisture content. Its relative humidity and temperature
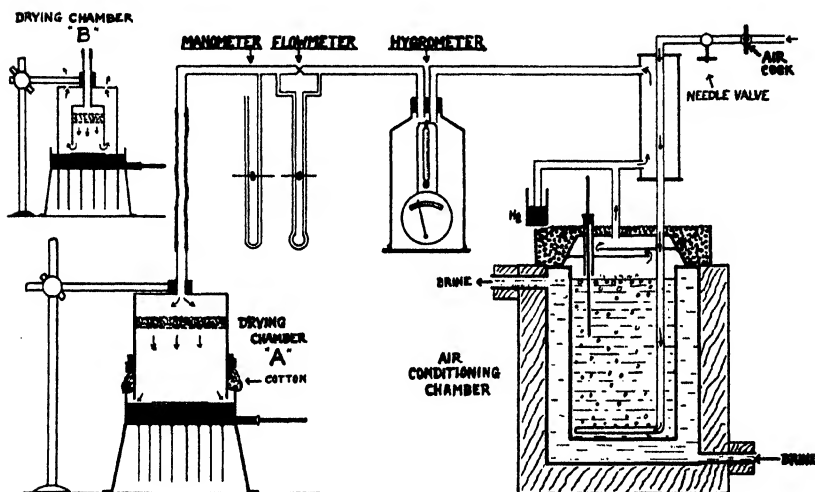
Fig. 1. Drying apparatus; alcohol membranes.

are measured by means of a hair hygrometer (Lambrecht polymeter), which has been standardized against a sling psychrometer. From the hygrometer the air passes through a flowmeter, calibrated in liters per minute, to the drying chamber. A water manometer shows whether there is any back pressure in the tube leading to the drying chamber. The drying chamber consists essentially of a metal dish containing mercury and fitted with a cover to protect its contents from the outside air. The mercury serves to level the glass plate which is floated upon it, and to conduct heat from the outside air to the plate as the collodion upon it evaporates. To aid the mercury in picking up heat from

the outside air a number of vertical radiator fins are soldered to the bottom of the metal dish. In Drying Chamber A the air passes through a diffusing septum of cotton near the top of the cover, down over the collodion on the glass plate, and out to the atmosphere through barriers of cotton wool. In Drying Chamber B the air passes through a cotton diffusing septum in an inner chamber which has approximately the same diameter as the glass plate, then over the collodion, and out through small holes in the top of the cover. The approximate temperature of the glass plate is measured by means of a thermometer in the mercury bath.

Other investigators have used plates of window glass and have had difficulty in removing membranes after water immersion. Nelson and Morgan suggest filming the cooled plate with moisture from the breath before pouring the collodion upon it, and while this procedure is efficacious as far as removing the film is concerned, it introduces into the collodion the factor of moisture with its undesirable properties of weakening the membrane and changing its permeability. The glass plates here employed are 7.5 cm. in diameter, with the sides ground parallel and one side highly polished. The plates were obtained from the Bausch and Lomb Optical Company. Membranes did not adhere to these plates, and the only cleaning necessary was to polish them with a soft cloth after the membranes had been removed in the immersion water.

Nitrocellulose solutions were made in liter quantities. They were allowed to stand for several days before using for two reasons: first, because perfect solution is not attained for some time after the collodion appears wholly liquid and homogeneous; second, to permit particles of dust and undissolved cellulose to settle to the bottom. Glass-stoppered bottles were used and the solutions protected from the moisture of the air by rubber caps covering the stoppers and necks. Pipettes were cleaned after use by rinsing several times with acetone and drying with suction.

The technique of making the membranes is simple. The air is turned on, regulated by the needle valve to a flow of 5 liters per minute, and allowed to pass through the apparatus for some time, until the hygrometer shows the proper degree of relative humidity and the whole apparatus is free from excess moisture. The cover

TABLE II.

Series I. Drying Chamber A.

4 cc. of 1 per cent ether-alcohol-parlodion solution on 7.5 cm. plate·
Dry weight of membrane, 0.0432 gm. (average of five samples).

Drying time.	Wet weight of membrane.	Water-nitrocellulose ratio (G_w).	Filtration rate.
min.	*gm.*		*cc. per sq. cm. per min.*
6	0.850	18.6	0.3360
8	0.810	17.8	0.3360
10	0.925	20.2	0.3540
12	0.790	17.3	0.2960
14	0.700	15.3	0.2870
16	0.610·	13.1	0.2700
18	0.770	16.8	0.2760
20	0.520	11.0	0.2230
22	0.450	9.3	0.1850
24	0.345	7.0	0.1570
26	0.330	6.6	0.1320
28	0.260	5.0	0.1170
30	0.250	4.8	0.1050

TABLE III.

Series II. Drying Chamber B.

4 cc. of 1 per cent ether-alcohol-parlodion solution on 7.5 cm. plate.
Dry weight of membrane, 0.0420 gm. (average of seven samples).

Drying time.	Wet weight of membrane.	Water-nitrocellulose ratio (G_w).	Filtration rate.
min.	*gm.*		*cc. per sq. cm. per min.*
18	0.382	8.1	0.1500
20	0.290	5.9	0.1300
22	0.235	4.6	0.1080
24	0.222	4.3	0.0950
26	0.171	3.1	0.0730
28	0.118	1.8	0.0570
30	0.106	1.5	0.0370
32	0.078	0.85	0.0200

of the drying chamber is then removed, the glass plate floated upon the mercury, and 4 cc. of the 1 per cent nitrocellulose solution pipetted upon the plate.   The cover is quickly replaced, and the collodion allowed to dry for the required time.   Grading is accomplished by varying the drying time.   When the required time

has elapsed, the cover of the drying chamber is removed, and the plate quickly lifted by means of a wire fork and immersed in water. The membranes of Series I (Table II) and Series II (Table III) were transferred directly to water in this manner. Those of Series III (Table IV) were transferred to 85 per cent alcohol, and carried through a series of alcohol-water mixtures of diminishing alcohol content to increase their permeability, as in the method of Nelson and Morgan.

When making a series of membranes, at the start of the run the

TABLE IV.

Series III. Drying Chamber B. Alcohol-Water Treatment.

4 cc. of 1 per cent ether-alcohol-parlodion solution on 7.5 cm. plate. Dry weight of membrane, 0.0420 gm. (average of seven samples).

Drying time.	Wet weight of membrane.	Water-nitrocellulose ratio (G_w).	Filtration rate.
min.	*gm.*		*cc. per sq. cm. per min.*
10	0.965	22.0	0.3525
12	0.807	18.4	0.3480
14	0.760	17.1	0.2820
16	0.650	14.5	0.3115
18	0.610	13.5	0.2860
20	0.550	12.1	0.2275
22	0.360	7.6	0.1805
24	0.340	7.1	0.1735
26	0.230	4.5	0.1315
28	0.205	3.8	0.1090
30	0.185	3.4	0.0800
32	0.110	1.6	0.0590
34	0.105	1.5	0.0220

temperature of the mercury in the drying chamber is that of the room. During the drying of the first membrane of the series, the mercury temperature falls 3–4°C. The first membrane is discarded. If little time elapses between the finish of drying one membrane and the start of drying the next, the mercury will maintain the lower temperature steadily through the series, provided the room temperature does not change materially.

The membranes of Series I, II, and III were made on a number of different days, experiments being conducted at such times as the room temperature could be maintained at approximately 21.5°C.

in order to obtain comparable results. The temperature of the drying air approximated that of the room, the mean being 21.5°C. with a variation of not more than 1° throughout all series of experiments. The mean temperature of the mercury in the drying chamber was 18.9°C. with a variation of not more than 2°. The relative humidity varied 1.5 per cent, about a mean of 22.5 per cent.

A number of preliminary experiments were made to determine the time of drying necessary to gell completely the collodion on the plate. Collodion, like shellac or enamel, dries first at the surface, leaving the underlying layer still liquid. If such a partially gelled film is immersed in water, a very permeable membrane is produced. In several instances membranes were obtained which had a water-nitrocellulose ratio of 27. Such membranes, however, are not even in thickness, and a series made by the same technique shows highly variable permeability. The point of complete gelling was recognized by rubbing the partly dried film with the finger. If gelling is complete, the film pulls away from the plate, leaving a clean glass surface. If not, a thin layer of liquid collodion is left upon the glass. For the 1 per cent nitrocellulose solution employed the time of complete gelling was, for Drying Chamber A, approximately 20 minutes; for Drying Chamber B approximately 14 minutes.

The water-nitrocellulose ratio (G_w) of the membranes was obtained by weighing the wet membrane, after blotting between filter papers, to the nearest half cgm., taking the average weight of a number of similar membranes which had been dried at 100°C. for 4 hours, and applying the formula:

$$G_w = \frac{\text{Wet weight} - \text{dry weight}}{\text{Dry weight}}.$$

The method employed for weighing the wet membranes is not very precise, for the amount of water removed by blotting depends upon the grade of the membrane, and upon the texture and moisture content of the filter paper, the pressure applied in blotting, and the loss of water from the membrane by evaporation during weighing, all variable factors which are difficult to control.

These membranes were used for ultrafiltration. The ultrafiltration apparatus is shown diagrammatically in Fig. 2. The sur-

face of the wire gauze is flush with the upper surface of the heavy brass base.   The opening in the base is approximately 5 cm. in diameter and the gauze is 16 mesh, made of wire 0.49 mm. thick, which leaves 8.44 sq. cm. of open space for filtration.   The membrane is placed upon the gauze, an annular rubber gasket laid
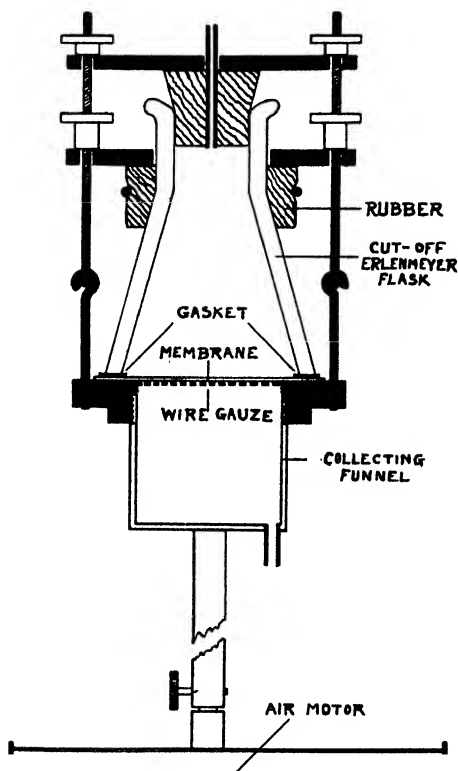
FIG. 2. Ultrafiltration apparatus.

upon it, and the cut off Erlenmeyer flask, the lower edge of which is ground plane, set in position on the gasket.   The filter cell so formed is held together by threaded rods and thumb nuts.   The brass base is threaded to make connection with the collecting funnel.   The whole apparatus is mounted on the vertical shaft of a reciprocating air motor (20) so that its contents may be shaken

during filtration. Shaking increases the rate of filtration of solutions, particularly those containing solid particles. Air pressure for driving the liquid through the membrane is obtained from a small tank fitted with a bicycle pump and a mercury manometer, and is applied to the contents of the cell through the tube in the stopper. A small thermometer, not shown in the diagram, is suspended inside the cell from a hook attached to the stopper. The cell holds about 80 cc. It is quickly assembled and easily taken apart, and will withstand a pressure of 1200 mm. of mercury without leaking.

The rate of filtration of distilled water under an air pressure of 400 mm. of mercury was taken as a basis for comparing the membranes of these series. The temperature of the room was maintained as nearly constant as possible. The time for filtering 50 cc. of water was noted, five such determinations being made upon each membrane. The filtration rate was expressed in cc. per sq. cm. of membrane per minute.

Tables II, III, and IV (Series I, II, and III) illustrate the characteristics of these membranes. In all three series it will be seen that the drying time is a better criterion of permeability than the water-nitrocellulose ratio. Series I and Series III show that the filtration rates of membranes dried for less than the time required for complete gelling have a wider distribution from the mean than those dried for a longer period of time. In Series I and II the relation between water-nitrocellulose ratio and drying time may be expressed by a straight line. In Series III, however, the line is a curve, due probably to the treatment of the membranes with alcohol-water mixtures to increase their permeability. It will be noted that even for membranes so treated the drying time is the better means of expressing permeability.

The hemoglobin of laked ox blood passed through a membrane dried in Chamber A for 32 minutes, but it did not pass through one dried 34 minutes. The hemoglobin passed through a membrane dried in Chamber B for 26 minutes, but not through one which was dried for 28 minutes.

From the results of these experiments one may conclude that if the constitution of the collodion, the condition of the drying air and its rate of flow, and the temperature of the drying film are held fairly constant, reproducible membranes may be made by

varying the drying time. If the films are dried for a longer time than is necessary to cause complete gelling of the collodion, the permeability of the resulting membranes is a straight line function of the drying time.

Although the method just described yields consistent results, it has one disadvantage; namely, the complication of the apparatus required. Therefore the research was continued with the idea of

TABLE V.

Series IV. Ethylene Glycol Membranes.

4 cc. of a 1 per cent ether-alcohol-parlodion solution containing ethylene glycol, on 7.5 cm. plate. Ether and alcohol evaporated completely in moisture-free atmosphere. Dry weight of membrane, 0.0390 gm. (average of five samples).

Ethylene glycol in collodion.	Ethylene glycol in 4 cc. collodion.	Ethylene glycol-nitrocellulose ratio.	Wet weight of membrane.	Weight of water in membrane.	Water-nitrocellulose ratio (G_w).	Filtration rate.
per cent	*cc.*		*gm.*	*gm.*		*cc. per sq. cm. per min.*
1	0.040	1	0.070	0.031	0.8	0.0175
2	0.080	2	0.115	0.076	1.95	0.1000
3	0.120	3	0.155	0.116	3.0	0.1400
4	0.160	4	0.195	0.156	4.0	0.2280
5	0.200	5	0.240	0.201	5.15	0.2500
6	0.240	6	0.280	0.241	6.2	0.3320
7	0.280	7	0.340	0.301	7.7	0.3340
8	0.320	8	0.365	0.326	8.35	0.3940
9	0.360	9	0.430	0.391	10.0	0.4225
10	0.400	10	0.500	0.461	11.8	0.4170
11	0.440	11	0.530	0.491	12.6	0.4050
12	0.480	12	0.520	0.481	12.3	0.4450
13	0.520	13	0.570	0.531	13.8	0.4890
14	0.560	14	0.615	0.576	14.8	0.5020
15	0.600	15	0.700	0.661	17.0	0.5190

evolving a simpler process. The essential determinant of the permeability of a membrane is the ratio of the solvent or swelling agent to the nitrocellulose in it at the moment of its immersion in water. Hence if one were to add a water-soluble, non-volatile swelling agent to alcohol-ether collodion, pour the mixture on a glass plate, and completely evaporate the alcohol and ether, the permeability of the membrane produced by immersing the plate

in water should be a function of the ratio between the non-volatile swelling agent and the nitrocellulose in the collodion.

In certain preliminary experiments along this line lactic acid was employed as the swelling agent and the films were allowed to dry in the open air. Lactic acid is hygroscopic, and the ordinary reagent contains 15 per cent of water; therefore membranes made under different atmospheric conditions gave inconsistent results when tested for permeability. After a number of experiments with various substances, ethylene glycol was chosen as the non-volatile swelling agent, and a simple apparatus was devised to allow evaporation of the volatile solvent in dry air.

Ethylene glycol is a colorless liquid having many of the physical characteristics of glycerol. The commercially pure substance (obtained from the Carbide and Carbon Chemicals Company) has a specific gravity of 1.1145 to 1.1178, an acidity of 0.005 per cent or less, and a water content of 0.5 per cent or less. It swells nitrocellulose, though its swelling power is much less than that of 95 per cent alcohol. A shred of dry parlodion 1.1 mm. thick was kept immersed in ethylene glycol for 6 weeks. At the end of that time it measured 1.71 mm. in thickness. The parlodion, after swelling, was very flexible and plastic. Parlodion kept in 95 per cent alcohol for 6 weeks became a soft gel.

The membranes of Series IV (Table V) were made from collodions containing ethylene glycol. The solvent mixture was prepared according to the formula:

	cc.
Ethyl alcohol, absolute	25
Ethylene glycol	0.5 to 15
Ether, Squibb's anesthetic, to make	100

In each 100 cc. of the solvent was dissolved 1 gm. of the dry nitrocellulose.

The drying apparatus is shown in Fig. 3. The collodion is pipetted onto glass plates floated on mercury, which is contained in metal dishes resting on a metal base plate. A metal cover makes an air-tight joint with the base plate by means of a petrolatum seal. A water aspirator draws a slow stream of air (about 10 cc. per minute) through a calcium chloride drying canister, through a hair hygrometer, and then through the film-drying chamber. A second

calcium chloride canister prevents the return of water vapor from the aspirator. The films are allowed to dry for 24 hours, which permits complete evaporation of the alcohol and ether. Upon immersion of the plates in water the membranes quickly float free. They are washed in running water for 48 hours to free them from the ethylene glycol, and stored in distilled water. Grading is
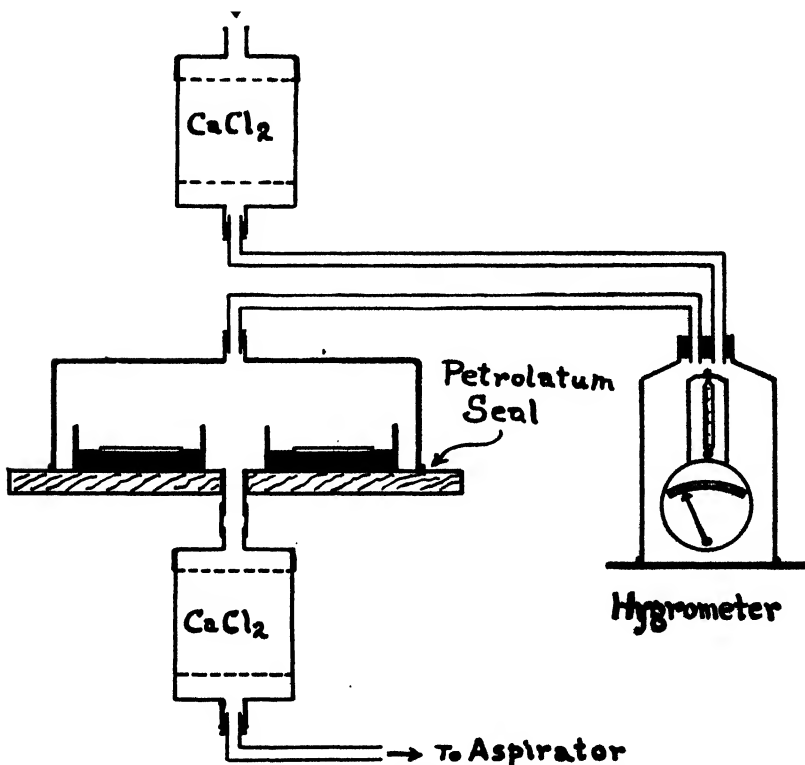
FIG. 3. Drying apparatus; ethylene glycol membranes.

accomplished by varying the percentage of ethylene glycol in the collodion, and thus the ratio between the volume of the swelling agent and the weight of the nitrocellulose in the film. The amount of swelling agent is expressed in terms of volume rather than weight because, as is shown in Table V, the swelling agent in the film is replaced by approximately its own volume of water. (The agree-

ment of the figures is good, considering the number of variable factors in the method of weighing the wet membranes. See p. 805.) The specific gravity of water being 1, the ratio between the volume of ethylene glycol and the weight of nitrocellulose in the collodion indicates the approximate ratio between weight of water and weight of nitrocellulose which may be expected to obtain in the finished membranes. It is necessary to conduct the drying process in a moisture-free atmosphere, for ethylene glycol is hygroscopic, and, as we have seen, water in the collodion weakens the membrane and changes its permeability.

The characteristics of these membranes are shown in Table V (Series IV). The filtration rate and the water-nitrocellulose ratio are straight line functions of the ethylene glycol-nitrocellulose ratio, up to an ethylene glycol-nitrocellulose ratio of approximately 6.5. Thereafter there is a wider distribution of the water-nitrocellulose ratios, and a change in the direction of the filtration rate curve.

The hemoglobin of laked ox blood passed through a Series IV membrane having an ethylene glycol-nitrocellulose ratio of 2.4, but it did not pass through one having a ratio of 2.0.

From a consideration of the foregoing experiments it is evident that membranes of graded permeability may be easily prepared by incorporating various percentages of a water-soluble, non-volatile swelling agent in anhydrous ether-alcohol collodion, evaporating the volatile constituents in a water-free atmosphere, and immersing the resulting gel in water. The grade of the membranes may be expressed in terms of the ratio between volume of non-volatile swelling agent and weight of nitrocellulose.

The author proposes the following theory as a possible explanation of the characteristics of nitrocellulose membranes. The nitrocellulose molecule is large and therefore may be said to have many valences (possibly partial or secondary) available for combination. In nitrocellulose in the solid state each molecule is in combination with adjacent molecules because of these valences or linkings. When nitrocellulose (e.g., a shred of parlodion) is treated with a swelling agent such as alcohol, some of these linkings are broken and combination with alcohol molecules takes place. Some of the linkings between adjacent nitrocellulose molecules still remain in place, hence the aggregation of nitro-
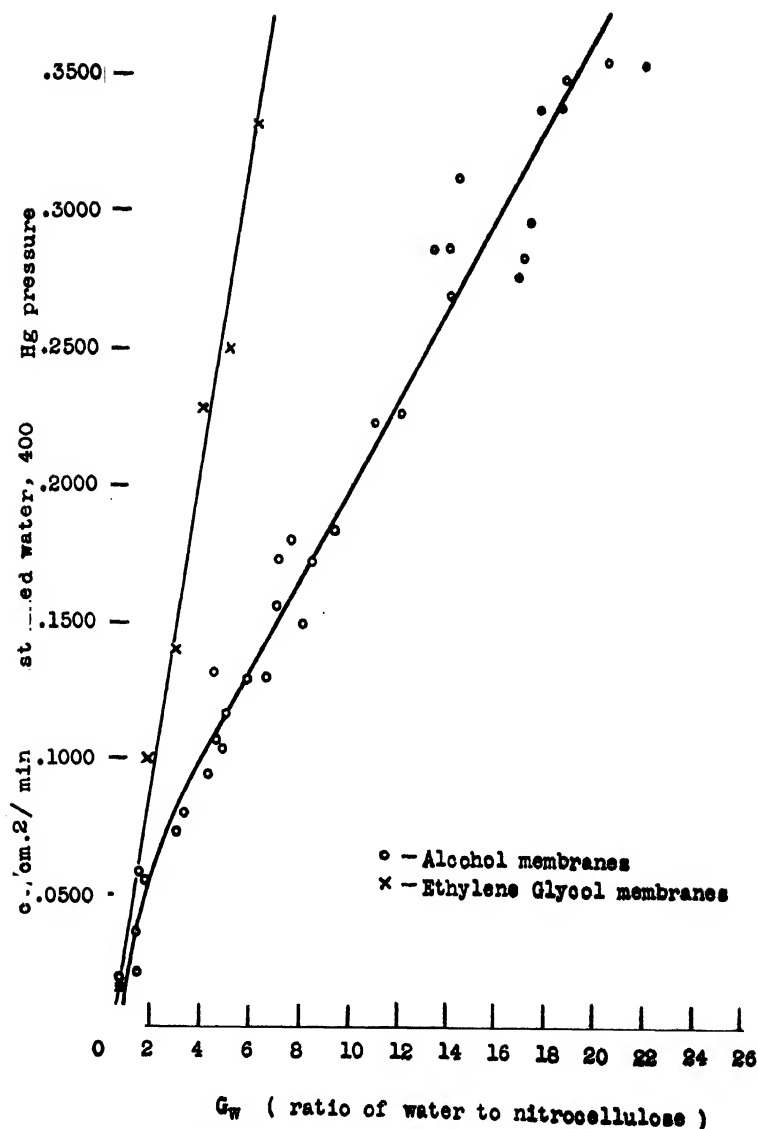
FIG. 4. Relation between water to nitrocellulose ratio and filtration rate; alcohol membranes and ethylene glycol membranes.

cellulose-alcohol complexes, or gel, retains something of the gross form of the original solid nitrocellulose. If ether is added to this gel, the remaining linkings between nitrocellulose molecules are broken, combinations between nitrocellulose, alcohol, and ether occur, and thus the gel loses its form and becomes a solution.

If from a concentrated solution made in this manner the ether is removed by evaporation, the nitrocellulose-alcohol-ether complexes are broken up, nitrocellulose-alcohol complexes are formed, and the sol becomes a gel. If from a highly dilute solution of nitrocellulose in ether and alcohol the ether is evaporated off, the result is not a gel but a suspension, the particles consisting probably of nitrocellulose-alcohol complexes. In this case, when the ether content falls so low that the mixture can no longer act as a solvent, the nitrocellulose molecules are so far separated by the relatively large numbers of alcohol molecules that the possibility of linking or combining with each other is prevented, with the result that a gel meshwork cannot form.

The structure, strength, uniformity, and permeability of a membrane depends upon the structure of the gel before immersion in water. If the ratio of solvent to nitrocellulose in the collodion employed is too high, no gel will be formed by drying, and the pasty mass of nitrocellulose and alcohol on the plate will either disintegrate when immersed in water or form a fragile membrane which cannot be removed intact from the plate. This explains Nelson and Morgan's observation that diluting the nitrocellulose solution beyond a certain point results in membranes which are too fragile to be used. It may also account for the lack of uniformity of those membranes of Series I and III which were immersed in water before complete gelling of the film had taken place, and for the wider distribution from the mean of the filtration rates of those Series IV membranes having an ethylene glycol-nitrocellulose ratio of more than 6.

Fig. 4 shows the relationship between the filtration rates and the water-nitrocellulose ratios of all of the membranes of the four series. It will be noted that for any given filtration time the water-nitrocellulose ratio is greater in the case of the alcohol membranes (Series I, II, and III) than in the case of the ethylene glycol membranes (Series IV). This is additional evidence that alcohol swells nitrocellulose more than does ethylene glycol.

SUMMARY.

1. A method of making nitrocellulose filtering membranes is described in which, by holding constant the constitution of the collodion, the temperature of the drying film, and the condition of the atmosphere in which the film is dried, reproducible membranes of different degrees of permeability may be prepared by varying the drying time.

2. A second and simpler method is described in which the permeability of the membranes is varied by incorporating different percentages of a water-soluble, non-volatile swelling agent in anhydrous ether-alcohol collodion, evaporating completely the volatile solvents in a water-free atmosphere, and immersing the resulting gels in water.

3. Evidence is brought to show that the prime determinant of permeability of a membrane is the ratio between the swelling agent or solvent and the nitrocellulose in the film at the time of its immersion in water.

4. A theory of membrane formation is proposed, and used to explain the characteristics of membranes prepared in this and other researches.

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